# MICROALGAL BIOMASS FRACTIONATION TO ENHANCE DOWNSTREAM BIOFUEL PRODUCTION

#### by

#### NAGA SIRISHA PARIMI

(Under the Direction of Keshav C. Das)

#### ABSTRACT

This work investigated microalgal/cyanobacterial protein fractionation to enhance biofuel production using wet biomass processing technologies (anaerobic digestion (AD) and hydrothermal liquefaction (HTL)). A method was developed for protein extraction from *Spirulina platensis* based on cell disruption, and a subsequent solubilisation and precipitation using alkali and acid respectively. At the optimized process conditions, extraction yield was 60.7 %. The obtained protein isolate had high protein content (80.6 %), and was enriched in essential amino acids and nutritional fatty acids, suggesting possible applications for human food or animal feed. The residual biomass had lower nitrogen and higher non-protein composition and was suitable for biofuel feedstock applications. AD of the protein extracted *S. platensis* residual biomass (PERB) resulted in 30.4 % higher methane yield than original (untreated) biomass. The rate of methane production was higher than that for original biomass (ORIB) and high pressure homogenizer disrupted biomass by 161 % and 38.9 % respectively. Biocrude oil produced from HTL of PERB was better in quality than that from ORIB owing to the presence of larger number of long chain hydrocarbons and fatty acids, and slightly lower nitrogen content (6.2 % versus 7.0

%). A comparison across AD and HTL suggested a better energy recovery for PERB in the former. Thereafter, the benefits of using protein extracted biomass residues generated from three different microalgal species (*Chlorella pyrenoidosa*, *Tetraselmis chuii and Phaeodactylum tricornutum*) by two different protein fractionation/ extraction methods (High pressure alkaliacid (HPAA) and low temperature hydrothermal treatment (LTHT)) were evaluated as feedstock for AD. HPAA method involved cell disruption and a subsequent protein extraction using alkali and acid. LTHT method involved low temperature hydrothermal treatment to extract proteins into the aqueous phase. Residues from the former method resulted in higher methane yields and methane production efficiencies than all other substrates of the respective species. The coproducts (protein isolates) had a composition suitable for food/ feed applications. LTHT method was beneficial only for the species with the most recalcitrant cell wall (*Chlorella pyrenoidosa*). Based on this work, a microalgal biorefinery may be proposed with the integration of protein extraction and biofuel production processes.

INDEX WORDS: Microalgae, Biofuel, Anaerobic digestion, Hydrothermal liquefaction, Protein extraction, Biorefinery

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## PRODUCTION

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# DEDICATION

To my parents and grandparents

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#### FOREWORD

This dissertation is aimed at developing processes for the fractionation and extraction of crude protein from microalgae/ cyanobacteria and the use of the residual biomass for biofuel production via wet processing technologies – anaerobic digestion (AD) and hydrothermal liquefaction (HTL). AD converts wet biomass to biogas, a gaseous fuel whose major component is methane. HTL converts microalgal biomass at high moisture content (80-90 %) to biocrude oil, which can be upgraded to petroleum-like hydrocarbon fuels. The extracted crude protein could find applications as human food or animal feed.

This dissertation is organized into 6 chapters. The first chapter provides a brief introduction on microalgae, discusses the limitations and proposed solutions for microalgal biofuel technologies, and finally leads to the specific objectives of this research work. The second chapter provides the background literature on AD, HTL and protein extraction methods that is highly relevant to this work. The third chapter reports the development and optimization of a method for extraction of proteins from *Spirulina platensis* cyanobacterium, and characterization of the product fractions for identification of bottlenecks of the process and the potential applications for the two product fractions. The fourth chapter reports the use of the residual biomass after protein extraction from *Spirulina platensis* as a substrate for AD and HTL and compares its performance relative to the untreated and cell disrupted (by high pressure homogenization) biomass. A comparison of the benefits on AD and HTL is also presented. The fifth chapter reports the use of biomass residues obtained after protein fractionation/ extraction using two different methods from three different microalgae. The performance of the fractionated residues is compared against the non-fractionated biomass for each of the species. A preliminary characterization of the co-product fraction from the most beneficial fractionation process is also presented. Finally, chapter seven presents the conclusions from this work and provides recommendations for future work in this direction.

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#### CHAPTER 1

#### INTRODUCTION

Fossil fuels have been the major source of energy worldwide since several decades owing to their high energy density, availability, wide applicability and relative cheapness. Currently, 80 % of the world's energy demand is met by fossil fuels (IEA, 2013). However, concerns over the depletion of fossil resources and the environmental impact of greenhouse gas emissions as a result of overdependence have driven interest in alternative sources of energy in the recent past. Anthropogenic uses of fossil fuels have resulted in  $CO_2$  emissions beyond 400 ppm and a further increase upto 960 ppm is projected (Dlugokencky & Tans; Joos et al., 2001). A limiting concentration of 500 ppm of atmospheric  $CO_2$  is expected to result in significant climatic impact (Pacala & Socolow, 2004).

Renewable sources of energy such as solar, hydro, wind and geothermal energy are being explored as an alternate to fossil fuels. However, these resources are capable of producing direct electricity alone. Biomass, on the other hand, is an attractive renewable resource for the production of solid, liquid and gaseous fuels which can be used for transportation and domestic fuel applications, in addition to electricity generation (Demirbas, 2011). By the year 2050, biomass is expected to provide upto 27% of world's transportation fuel (IEA, 2011). Use of biomass as an energy source has the advantages of ensuring energy security, CO<sub>2</sub> mitigation and foreign exchange savings (Demirbas, 2008). However, the sustainability of biofuel technologies are limited by low net energy output, slow growth rates of biomass resources, high costs of production and processing and the increasing concern over the competition for land and water resources with food crops (Mussgnug et al., 2010; Yu et al., 2011).

Microalgae are perceived as a reliable biomass resource for the production of biofuels and valuable co-products due their high productivity, high growth rate and photosynthetic activity,  $CO_2$  mitigation, ability to grow on non-arable land using wastewater, saline and brackish waters, and the ability to accumulate high oil content (upto 50%) (Borowitzka & Borowitzka, 1990; Brown et al., 2010; Chisti, 2007; Mussgnug et al., 2010; Singh & Gu, 2010). They can be converted to biofuels through various thermochemical and biochemical processes such as pyrolysis, liquefaction, gasification, fermentation and anaerobic digestion (Demirbas, 2011). However, microalgal technology has the limitations of high costs of production, harvesting and dewatering, and significant variation of composition owing to numerous environmental, growth and physiological factors (Becker, 1994; Molina Grima et al., 2003). These limitations have to be overcome to make microalgal biofuels sustainable.

Harvesting of microalgae is a highly energy intensive process (Molina Grima et al., 2003). Most of the traditional harvesting methods result in algal biomass with high moisture content (about 80-90 %), necessitating the need for further dewatering in order to be utilized as feedstock for biofuel conversion processes. This further increases the cost exponentially. Thus, wet processing technologies capable of directly converting microalgal biomass at high moisture content to biofuels can potentially ensure sustainability and cost-effectiveness. Hydrothermal liquefaction (HTL) and anaerobic digestion (AD) are such technologies (McKendry, 2002; Patil et al., 2008).

Hydrothermal liquefaction is the thermochemical process of converting biomass to crude oil-like liquid fuel (biocrude oil) by hot compressed water. Complex compounds such as carbohydrates, lipids and proteins present in biomass decompose and undergo a serious of reactions to yield the final product with a higher energy density compared to the feed biomass (López Barreiro et al., 2013). The biocrude oil thus generated can be upgraded to transportation fuels. Compared to other thermochemical technologies, HTL has the benefits of processing wet biomass, enhanced reaction rates, efficient product separation, simultaneous generation of useful co-products and nutrient recycle (from the aqueous phase) (Jena et al., 2011; Peterson et al., 2008). However, a major drawback of microalgal HTL is the presence of high protein content in the feedstock resulting in a biocrude with undesirably high nitrogen content that could potentially lead to detrimental effects such as acid rains due to the formation of toxic oxides of nitrogen (NOx) upon combustion (López Barreiro et al., 2013).

Anaerobic digestion is the biological process of converting organic materials into biogas by anaerobic bacteria. Organic polymers such as carbohydrates, lipids and proteins are hydrolyzed and broken down into monomers which are then converted to biogas by fermentation process (Parmar et al., 2011). Methane and  $CO_2$  are the major components of biogas. The methane gas obtained by scrubbing  $CO_2$  off biogas may be used in place of natural gas for transportation, domestic fuel and electricity generation (Bohutskyi & Bouwer, 2013). The digestate can be used for nutrient recycle as a fertilizer (Phang et al., 2000). One of the major limitations of microalgal AD is the high cell wall recalcitrance, resulting in slower hydrolysis kinetics, lower biomass conversion, lower yields and higher energy inputs (Bohutskyi & Bouwer, 2013). Another major limitation is the high protein content of microalgae resulting in the formation of ammonia at inhibitory concentrations and the contamination of biogas (Chen et al., 2008; Strik et al., 2006). The above mentioned limitations for AD and HTL may be overcome by microalgal biomass fractionation and protein extraction. Component fractionation/extraction methods usually involve pretreatments that can partially or completely degrade the cell walls and increase access to intracellular components. Extraction of intra-cellular proteins would result in decreased nitrogen content and increased carbon content of the microalgal feedstock. Further, microalgal proteins have a high nutritive value (Becker, 2007). Thus, an added merit of extracting microalgal proteins is the potential utility of this fraction as a nutritional supplement for humans and/or feed for animals. This approach could help mitigate the food insecurity problem that is threatening the world today.

The current research study was aimed at investigating pretreatments and protein fractionation methods for microalgae (cyanobacteria included), and studying their impact on downstream biofuel production via AD and HTL processes. Since the cell wall structure and biochemical composition of microalgae differ significantly across different species, some of the studies were carried out across multiple species. The specific objectives of this study were to:

- 1. Develop a process for protein extraction from microalgae/cyanobacteria, optimize process parameters for maximizing extraction yield, identify bottlenecks in the process, and characterize the obtained protein isolate and the residual biomass to identify potential applications.
- 2. Evaluate the performance of the residual biomass obtained after protein extraction at the optimized process conditions as feedstock for biomethane production via AD and biocrude oil production via HTL, and compare the two processes.
- 3. Evaluate and compare the benefits of using protein extracted biomass residues (obtained by two different fractionation/extraction methods) as feedstock for AD and compare the impact

across different microalgal species that differ significantly in cell wall structure and biochemical composition.

In the long run, it is expected that the concepts established through this research will help in building sustainable microalgal biorefineries that can simultaneously produce protein supplements for human/ animal consumption and biofuels.

A brief review of literature in the areas related to this dissertation is presented in Chapter 2.

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#### CHAPTER 2

#### LITERATURE REVIEW

#### 2.1. Introduction

Microalgae are attractive biomass resources that can help mitigate the global challenges of food and energy insecurity through integrated biorefinery approach (Subhadra, 2011). They are versatile microorganisms that do not compete with food crops for land resources, can grow in saline, brackish and waste waters and are rich in oil and protein contents (Parmar et al., 2011). However, microalgal biomass processing technologies are limited by their high moisture content (> 90%) that necessitates the use of expensive drying and dewatering steps (Molina Grima et al., 2003). Thus, the need for research and development on processing technologies that are capable of converting wet microalgae to biofuels and co-products is paramount. This review focuses on the current state of the art knowledge on wet algae biomass to biofuel conversion technologies (anaerobic digestion (AD) and hydrothermal liquefaction (HTL)), their limitations and research solutions that were explored in literature.

#### 2.2. Anaerobic digestion

Algae biomass as substrates for AD was first explored by Golueke *et.al.* (1957) (Golueke et al., 1957). Presently, microalgae and cyanobacteria are regarded as attractive substrates for biogas/ biomethane production, as is evident from the drastic increase in the number of publications in this area in the recent past.

Anaerobic digestion is a biological process in which microorganisms (bacteria and archaea) degrade organic compounds such as proteins, lipids and carbohydrates present in biological matter under anoxic environment to produce biogas. The main components of biogas are methane and carbon dioxide, although small amounts of ammonia, hydrogen sulfide, hydrogen and volatile organic compounds are often present. Biogas can either be used directly as a fuel or upgraded to biomethane that can be used in place of natural gas for transportation, electricity generation and other domestic and industrial applications (Bohutskyi & Bouwer, 2013).

AD is a highly complex process that involves four stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Chynoweth, 1987). Hydrolysis is the process of breaking down complex organic polymers into soluble compounds, and is usually the rate limiting step in the AD of a wide range of organic substrates (González-Fernández et al., 2011). Acidogenesis is the conversion of these soluble polymers into organic acids, alcohols, H<sub>2</sub> and CO<sub>2</sub>. Acetogenesis is the process of converting the volatile fatty acids and alcohols into acetic acid and hydrogen. Methanogenesis is the process of conversion of acetic acid and hydrogen to methane and carbon dioxide. The first two stages are carried out by fermentative hydrolytic bacteria, the third by hydrogen producing bacteria (and archaea), and the last by methanogeneic bacteria (and archaea).

AD of biomass has several advantages. First, it is not a very energy intensive process and does not require prior concentration, drying, oil extraction or nutrient supplementation. Second, unlike other biofuel technologies such as production of biodiesel and bioethanol, all the organic

macromolecules can be converted to biogas. Third, the digestate from AD can be used for nutrient recycle (González-Fernández et al., 2011).

#### 2.2.2 Methane yields

#### Theoretical methane yield

The maximum capacity of a substrate for methane production, the theoretical methane yield, can be estimated based on the elemental composition of the substrates according to the modified Buswell and Mueller stoichiometric equation as shown below (Buswell & Mueller, 1952; Gallert et al., 1998; Richards et al., 1991):

$$C_{n}H_{a}O_{b}N_{c} + (n - 0.25a - 0.5b + 1.75c)H_{2}O$$

$$\rightarrow (0.5n + 0.125a - 0.25b - 0.375c)CH_{4}$$

$$+ (0.5n - 0.125a + 0.25b - 0.625c)CO_{2} + cNH_{4}^{+} + cHCO_{3}^{-}$$
(1)

#### Biochemical methane potential

Biochemical methane potential (BMP) is a measure of the experimental maximum methane yield that can be obtained from a substrate based on its volatile solids (VS) content (Hansen et al., 1998). From this value, the biodegradability (the percentage ratio of BMP to theoretical maximum yield) of a substrate can be determined. BMP tests are conducted as batch experiments and the data obtained can give useful information on substrate degradation kinetics, inhibition studies and comparative evaluation of different substrates for methane production (Nallathambi Gunaseelan, 1997). The parameters which affect experimental results are pH, stirring intensity, physical and chemical characteristics of the substrate and substrate/inoculum ratio. A major disadvantage of BMP is that it cannot give valuable information about variation of substrate digestibility, process kinetics and process efficiency with time, unlike a continuous AD reactor (Ehimen et al., 2011). Several authors have used BMP as a method to compare methane production from different microalgal substrates (Alzate et al., 2012; Mendez et al., 2014; Schwede et al., 2011; Zamalloa et al., 2012).

#### 2.2.3 Parameters impacting microalgal AD

The following process parameters are known to impact microalgal AD:

- Temperature: Temperature impacts the rate and extent of methanogenesis. The rate of methane production is the least at psychrophilic conditions (10-15 °C) and the highest at thermophilic conditions (50-55 °C). Higher algae digestion at thermophilic conditions than mesophilic conditions was reported previously (Golueke et al., 1957). However, another study reported no significant difference in biogas production rates during the digestion of *Scenedesmus obliquus* and *Phaeodactylum tricornutum* in hybrid flow-through reactors under the two conditions (Zamalloa et al., 2012). Moreover, AD at thermophilic conditions is usually more sensitive than mesophilic conditions to inhibitory effects such as ammonia toxicity that can lead to process failure (Parkin & Miller, 1982). Thus, mesophilic conditions are generally preferred.
- 2. *pH*: pH is a significant factor impacting AD. The optimum pH for acidogens is 5.5-6.5 and methanogens is around 7.8-8.2 (Khanal, 2011). Generally, pH of AD is maintained in the range of 7-8 owing to the higher sensitivity of the latter. pH also controls the speciation of carbonates and the release of CO<sub>2</sub>. Higher pH results in higher alkalinity, lower CO<sub>2</sub> and higher methane in the produced biogas (Sialve et al., 2009; Singh & Olsen, 2011).
- 3. *Hydraulic retention time (HRT) and organic loading rate (OLR):* HRT is the time for which substrates stay in the AD reactor. OLR is a parameter that determines the biological working

capacity of the AD reactor. HRT and OLR are optimized based on the type and composition of the algal biomass to prevent washout and accumulation of toxic inhibitors (Sialve et al., 2009). These parameters are applicable to continuous or semi-continuous reactor systems. In batch AD, the input organic load (gVS or gCOD) and digestion period are considered.

*Toxicity:* High concentrations of ammonia, alkali metal ions, sulfide ion and other inhibitors 4. such as cyanotoxins impact AD. Ammonia is produced by the biological degradation of proteins. In small concentration (< 200 mg/ L), it is beneficial for the anaerobic microorganisms since it is required for cell metabolism and protein synthesis. However, at high concentrations (1.5 - 14 gN/L), it inhibits AD (Chen et al., 2008). Ammonia could either be directly detrimental for the bacteria or it could bring about a drastic change in substrate pH to the level intolerable by the methanogens (Snell, 1943). Mechanisms of ammonia inhibition include passive diffusion of unionized free ammonia through bacterial cell walls and alteration of intracellular pH, potassium deficiency, increase of maintenance energy requirement and inhibition of any enzymatic reactions (Gallert et al., 1998; McCarty, 1964; Sprott & Patel, 1986; Wittmann et al., 1995). Ammonia inhibition is commonly observed in microalgal AD. It was reported that Spirulina maxima containing 60 % protein releases inhibitory concentration of ammonia (7 g/L) (Samson & Leduyt, 1986). Another study reported ammonia inhibition in a thermophilic reactor digesting Nannochloropsis residues (obtained after dry-extraction of oil) (Kinnunen et al., 2014). The inhibitory concentration of ammonia depends on the nature of inoculum, operating conditions and acclimatization levels (Angelidaki & Ahring, 1993). Thermophilic conditions are more sensitive to ammonia relative to mesophilic conditions (Parkin & Miller, 1982).

Acclimatization improves the toxicity threshold. pH influences the speciation of ammonium ion and free ammonia, the latter has a higher toxicity than the former (Ehimen et al., 2011).

Alkali metal ions such as sodium, calcium and potassium which increase the alkalinity, impact AD by having an antagonistic effect on ammonia inhibition (Chen et al., 2008). Sodium ion concentrations of 0.14 M and above are reported to lead to toxicity (McCarty, 1964). For example, a lower methane yield was observed for the marine strain *Dunaliella tertiolecta* (24 ml gVS<sup>-1</sup>) relative to the fresh water strain *Chlorella vulgaris* (286 ml gVS<sup>-1</sup>) (Lakaniemi et al., 2011). Sulfur containing amino acids present in proteins can release hydrogen sulfide that can be toxic to methanogens at high concentrations. However, sulfate toxicity is not of great concern in the case of microalgae due to their low sulfur content (Becker, 1988). In addition, AD can be inhibited by other toxic compounds such as cyanotoxins, N-substrituted aromatics and halogenated aliphatics. The completely degraded *D.salina* and *A.platensis* substrates yielded less biogas than *C. reinhardtii*, suggesting the presence of toxic inhibitory compounds (Mussgnug et al., 2010).

5. Inoculum characteristics and concentration: Nature of inoculum, acclimatization period and inoculum concentration relative to substrate (I/S) impact AD. Acclimatization helps in improving toxicity threshold (Chen et al., 2008). High inoculum concentration (I/S ratio) helps in maintaining pH and avoiding destabilization of AD process (Nallathambi Gunaseelan, 1997). Alzate et al., 2012 studied the effect of inoculum to substrate (I/S) ratios on 3 different mixed culture microalgae and observed the highest methane productivities at I/S ratio of 2:1, irrespective of the microalgal mixtures (Alzate et al., 2012). The decrease in productivity at I/S ratio of 1:1 was very low (9-11 %) but was reasonable at I/S ratio of 1:3.

#### 2.2.4 Strain variability and limitations of AD

In addition to the process parameters mentioned in section 2.2.3, microalgal AD is heavily influenced by strain characteristics such as cell wall structure and biochemical composition (González-Fernández et al., 2011). The structural characteristics of microalgae range from extremely recalcitrant cell walls composed of insoluble polysaccharides (cellulose, hemicellulose, algaenan, sporopollenin) to more fragile glycoprotein based cell walls and in some cases lack of cell walls (Bohutskyi et al., 2014). Generally, species with more recalcitrant carbohydrate based cell walls result in lower methane yields than those with protein based cell walls or those that lack cell walls. For example, higher methane yields (0.4-0.5 L/ gVS) were observed for Tetraselmis sp., Pavlova\_cf sp. and Thalassiosira weissflogii as opposed to Nannochloropsis sp. and Chlorella sp. (~0.35 L/ gVS) (Bohutskyi et al., 2014). Another study reported higher yield for the wall-less Chlamydomonas reinhardtii than the polysaccharide containing Scenedesmus obliquus (Mussgnug et al., 2010). In another study, methane yield from Phaeodactylum tricornutum (0.36 L CH4/ gVS) was higher compared to Scenedesmus obliquus (0.24 CH4/ gVS) (Zamalloa et al., 2012). Thus, cell wall recalcitrance is a major limitation to microalgal AD.

Algae are comprised of proteins, carbohydrates and lipids. The theoretical maximum methane that can be produced from each of these components is:  $1.014 \text{ L CH}_4/\text{ gVS}$  for lipids,  $0.851 \text{ L CH}_4/\text{ gVS}$  for proteins and  $0.415 \text{ L CH}_4/\text{ gVS}$ . The theoretical maximum methane productivity for each microalgal species varies based on the contents of each of these components (Sialve et al., 2009). Lipids are carbon rich compounds and proteins are the nitrogen sources. The high protein content of microalgae is a major limitation owing to possible inhibition and toxicity from ammonia formed by protein degradation. The contamination of biogas with

ammonia is also unfavorable as it prevents its direct use as a fuel due to the possibility of harmful NO<sub>x</sub> emissions, and also adds to the costs involved in upgrading to biomethane (Strik et al., 2006). Thus low protein content or a higher C/N ratio is desired to overcome these limitations. Microalgae have a C/N ratio < 10, which is below the optimum range of 20-30 (Parkin & Owen, 1986).

#### 2.2.5 Pretreatments to enhance AD

Several pretreatments were explored in literature to enhance methane yields and anaerobic digestibility of microalgae. Some of them include mechanical, thermal, theromochemical, hydrothermal, chemical and enzymatic. The first 4 treatments (summarized in Table 2.1) are more widely used owing to their effectiveness for common microalgal species such as *Chlorella* and *Scenedesmus* with a recalcitrant cell wall, although the impact varied depending on operating conditions. Chemical treatments were not found to be very effective in enhancing methane yields (Bohutskyi et al., 2014; Mendez et al., 2013). It was reported that enzymatic pretreatment enhanced methane yields (Ehimen et al., 2013). However, the literature on enzymatic pretreatments is still very scarce and hence is not conclusive. Pretreatments work by causing cell rupture, increase of cell wall permeability, hydrolysis of cell polymers and deactivation of toxic materials (Bohutskyi & Bouwer, 2013; Sialve et al., 2009). As most pretreatments have a high energy requirement, the benefits and energy balance for the pretreatment coupled AD process should be carefully evaluated to ensure process sustainability.

#### 2.2.6 AD of microalgal residues obtained after component fractionation

Lipids are high energy molecules and have the highest theoretical methane capacity (Sialve et al., 2009). However, due to their low alkalinity and buffering capacity, they can become inhibitory to AD processes, especially when their content exceeds 31 % (Cirne et al.,

2007; Ward et al., 2014). Thus, lipid extraction is recommended prior to AD, if present in high amounts (>40 %). The extracted lipids can be used for the production of liquid fuels such as biodiesel and bio-oil. The spent biomass after lipid extraction can be used for biomethane production. Some studies explored the use of the lipid/ oil-extracted algae (LEA) as substrates for AD. Methane yields of 482 L/ kgVS and 194 L/ kgVS respectively were reported from batch AD of Nannochloropsis residues obtained after oil wet-extraction and dry-extraction respectively. In semi-continuous mode, Nannochloropsis residues after dry oil-extraction produced 48 % higher methane yield at thermophilic conditions than at mesophilic conditions (Kinnunen et al., 2014). The literature on the performance of LEA relative to the non-extracted algae is not conclusive as different outcomes were observed in different works. For example, one study reported higher methane yields for the non-extracted Chlorella sp. relative to LEA obtained by 1-butanol extraction and acid catalyzed in situ transesterification method (Ehimen et al., 2009). The LEAs obtained from these methods also showed a small difference in methane yields. In another study no significant difference in methane yields was observed for the nonextracted and lipid extracted *Phaeodactylum tricornutum*, although the LEA from the other species used in that study showed lesser methane compared to non-extracted biomass (Zhao et al., 2014). In yet another study, extraction of lipids from *Tetraselmis sp.* by supercritical  $CO_2$ method enhanced methane yields for the residual biomass relative to untreated by 47.5% (Hernández et al., 2014). Thus, the benefits of the use of lipid extracted residues for AD seem to depend on the lipid extraction method.

On the other hand, the performance of protein or amino acid extracted substrates was consistent among the few studies that were reported. Higher methane yield (enhancement by about 94.4 %) and a higher anaerobic digestibility relative to the untreated *Scenedesmus sp.* 

biomass was reported for residues obtained after amino acid extraction by enzymatic hydrolysis method (Ramos-Suárez & Carreras, 2014). These residues also performed better than lipid extracted residues in that study. In a continuous reactor study by the same authors, higher biogas yields were observed from amino acid extracted residues than untreated biomass of *Scenedesmus sp.* (Ramos-Suárez et al., 2014). Another study reported an enhancement of methane yield by *Scenedesmus sp.* residues obtained after protein extraction by free nitrous acid (FNA) pretreatment method (Astals et al., 2015).

#### 2.3 Hydrothermal liquefaction

#### 2.3.1 Theory

Hydrothermal liquefaction is the process of converting biomolecules such as lipids, proteins and carbohydrates into biocrude oil, gases, water soluble and solid products in hot compressed water (200 – 370 °C and 4-21 MPa). Water under subcritical conditions serves as a highly reactive solvent medium owing to a change in physicochemical characteristics such as density, polarity, dielectric constant, acidity and solubility, and hence is capable of mediating a series of chemical reactions that do not occur in normal water. The specific mechanisms identified for HTL are as follows: (i) Hydrolysis of macromolecules into smaller molecules; (ii) cleavage, dehydration and decarboxylation; (iii) repolymerization and recondensation to form high molecular weight compounds (Peterson et al., 2008; Toor et al., 2011).

The biocrude oil generated from HTL is composed of hydrocarbons and O/N-containing compounds, and can potentially be co-refined with fossil fuels to produce liquid fuels and chemicals (Tian et al., 2014). The HTL gas phase is usually composed of  $CH_4$ ,  $CO_2$ , CO,  $C_2$ - $C_5$
(Brown et al., 2010; Jena et al., 2011a). The aqueous phase is composed of organics and minerals which may be used for nutrient recycle. The solid phase primarily comprises of inorganics (Guo et al., 2015).

# 2.3.2 Advantages and limitations of HTL

HTL is an attractive option for converting microalgae to liquid fuels due to: (1) its ability to process wet microalgae (10-25 % solids) which can be obtained from direct harvesting, thereby eliminating the necessity for additional drying and dewatering steps; (2) ability to convert carbohydrates and proteins present in microalgae, in addition to lipids to a liquid fuel intermediate (biocrude oil); (3) easy separation of liquid, solid and gaseous products; (4) recycle of nutrients such as nitrogen, phosphorus and minerals (Guo et al., 2015; Tian et al., 2014).

The contribution of each of the microalgal cell components to biocrude yield is of the order lipid > protein > carbohydrate (Sawayama et al., 1999). However, subjecting proteins to HTL is disadvantageous as it results in a biocrude with a high nitrogen content (López Barreiro et al., 2013). This nitrogen is usually present in the form of long chain aliphatic amines and amides, and nitrogen heterocyclics (such as pyrroles, pyridines, indole etc.) (Biller & Ross, 2011; Brown et al., 2010; Jena et al., 2011a). These compounds are formed as a result of deamination, decarboxylation and cross reactions of amino acids (obtained from protein hydrolysis) with carbohydrates and lipids (Brown et al., 2010; Sato et al., 2004). The presence of nitrogen in the biocrude oil poses a serious problem to the upgrading processes due to the possibility of catalyst poisoning and deactivation, and the increased hydrogen demand during hydrodeoxygenation (Ross et al., 2010).

## 2.3.3 Parameters impacting microalgal HTL

The following process factors are known to impact biocrude yields and quality:

- 1. Temperature: HTL is heavily influenced by operating temperature. Many studies reported operating temperatures in the range of 300-350 °C to be favorable for microalgal HTL. Increase in temperature in the subcritical region promotes the conversion of intermediate water soluble products. Biocrude produced at higher temperatures was found to have a higher HHV (Garcia Alba et al., 2011). However, a higher temperature also causes degradation of proteins, resulting in high nitrogen content in the biocrude. A further increase of temperatures beyond the critical point results in increase in gas yield and decrease in oil yield (Guo et al., 2015).
- 2. Holding time: Reaction time is the time after attaining the maximum temperature without taking into account the heating and cooling periods. It affects product distribution of HTL and hence is a critical factor. A range of 30-60 min has been suggested for obtaining maximum bio-oil yield in HTL (Tian et al., 2014). However, high yields at shorter holding times were also reported (Eboibi et al., 2014; Valdez et al., 2012). Thus, holding time seems inversely related to temperature i.e. higher temperature operations require lower holding times and vice versa to carry out the reactions. In one study, HTL at 280 °C for 120 min resulted in a biocrude yield of 39.4 % (Yu et al., 2011). In another study, HTL at 375 °C for 5 min resulted in a biocrude yield of 49.4% (Garcia Alba et al., 2011).
- Catalyst: Catalysts improve biocrude yields and decrease the solid residue content. The use of both homogenous catalysts such as Na<sub>2</sub>CO<sub>3</sub> and KOH, and heterogeneous catalysts such as Pt, Co, Ni, Ni/Al, Pt/Al, Co/Mo has been reported in literature (Biller et al., 2011; Duan & Savage, 2011; Ross et al., 2010; Shuping et al., 2010; Yang et al., 2010). Homogenous

catalysts increase the pH and facilitate decarboxylation reactions while inhibiting dehydration reactions. A major drawback of catalytic HTL is the difficulty of recovering the catalyst. Heterogeneous catalysts can be easily recovered from the products; they help in denitrogenation and significantly improve biocrude yields. However, they have the limitations of sintering, poisoning or intraparticle diffusion (López Barreiro et al., 2013).

#### 2.3.4 Methods to reduce HTL biocrude nitrogen

Maio et al., 2014 developed two stage HTL method for yeast, where sugars and proteins were extracted in the first stage and the residual biomass was converted to bio-oil in the subsequent stage at 240 °C. The optimum temperature for the first stage to obtain maximum sugar and protein extraction with the minimal formation of inhibitory compounds was found to be 180 °C. At these conditions they observed that the biocrude quality was better owing to its lower nitrogen and higher fatty acid contents (Miao et al., 2014). Costanzo et al., 2015 also employed a low temperature hydrothermal pretreatment step before a subsequent high temperature HTL to reduce the biocrude nitrogen of 3 sets of microalgal cultures. They found higher nitrogen removal when pretreatment was carried out at 225-250 °C and holding times 5-15 min. They were able to remove about 45-65 % of the nitrogen present in algae solids (depending on the feedstock strains) using the pretreatment, affecting a slight reduction of biocrude nitrogen and an improved biocrude quality (Costanzo et al., 2015). Jazrawi et al., 2015 performed a two stage HTL at temperatures < 200 °C for stage I and 250-350 °C for stage II to reduce the biocrude nitrogen. Maximum amount of nitrogen (54 %) was extracted from stage I when pretreatment was carried out at the highest temperature (200 °C), although a significant reduction of solid yield was also observed. Yet, the least biocrude nitrogen was observed when pretreated at this temperature (Jazrawi et al., 2015). One study reported the technical evaluation of a biorefinery approach where the residual biomass after lipid extraction by Soxhlet method using n-hexane and a subsequent protein extraction by enzymatic hydrolysis, was subjected to HTL to produce biocrude oil (López Barreiro et al., 2014). The extracted proteins were transformed into valuable amino acids. The authors suggested that this route, without the lipid extraction step, is promising to obtain biocrude at a higher yield and lower nitrogen content.

#### 2.3.5 Nutrient recycle

The aqueous phase from the HTL process can be separated and recycled for algal growth. Several authors studied microalgal growth using the nutrients from this co-product and suggested that the composition is suitable for cultivation upon dilution, and the tolerance levels to inhibitors varies with species (Biller et al., 2012; Garcia Alba et al., 2013; Hognon et al., 2015; Jena et al., 2011b). The CO<sub>2</sub> and NH<sub>3</sub> gas present in the gas phase can also be recycled for microalgae cultivation. Nutrient recycle is essential for improving the sustainability of microalgal HTL (Tian et al., 2014).

# 2.4 Microalgal protein extraction

Table 2.2 summarizes the literature on microalgal protein extraction methods that were explored in literature. Among them, the pH shifting method using alkali and acid is popular. This method is based on the principle of increase in protein solubility at high and low pH due to a net charge acquired by them, leading to electrostatic repulsions. At the isoelectric point, the net charge becomes zero and the proteins precipitate out (Wheelwright, 1994). The advantages of this method are the low cost of reagents, scalability of the process and non-toxicity, since the acid and alkali can be eventually neutralized to salts (Ingadottir, 2004). The disadvantages are

the possibility of protein denaturation (at high temperatures and adverse pH (pH < 2 and pH > 12), formation of emulsions in the presence of lipids, and the low yields when carried out without a prior cell disruption step (Omana et al., 2010). Some microalgae such as *Chlorella*, *Scenedesmus* and *Nannochloropsis* have a highly recalcitrant cell wall necessitating a prior cell disruption technique to improve protein extraction yields (Ursu et al., 2014). In this context, the various cell disruption methods used include high pressure homogenization, sonication and bead milling. More recently, pulsed electric field as a method to perforate cells to enhance selective extraction of intracellular metabolites was reported (Parniakov et al., 2015).

#### 2.5 Microalgal biorefineries

Microalgal biorefineries are aimed at the simultaneous production of biofuels and high value products to improve the economics and overall sustainability of microalgal technologies. Several high value products can be extracted from microalgae such as proteins, pigments, omega-3 fatty acids (DHA, EPA), bioactive compounds, etc (Samarakoon & Jeon, 2012; Spolaore et al., 2006). Some of these products such as astaxanthin and beta carotene are commercially available.

Microalgal proteins have a high nutritive value and hence find applications as human food supplements, animal feeds and in aquaculture (Spolaore et al., 2006). A study on microalgae as a source of protein based on amino acid composition, digestibility coefficient and biological value inferred that they are suitable for food and feed applications (Becker, 2007). Some microalgal proteins are also suitable for cosmetological and pharmaceutical applications (Olaizola, 2003). A techno-economic analysis study of protein extraction from *Chlorella fusca* based on alkali and enzymatic method estimated the cost of protein extract at  $\notin$  2448/ ton using alkali method and  $\in$  1367/ ton using enzymatic method, both of which are far from industrial feasibility. The authors recommended the use of the residue after protein extraction for biofuel production as one of the means to enhance the revenue (Sari et al., 2016). This dissertation explored this idea.

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Table 2.1 Methane yield enhancement by various pretreatments for different microalgae in batch

AD/ BMP tests

Species	Pretreatment conditions	Methane yield enhancement (%)	References	
Thormal protroatmonts				
Scenedesmus	70, 90 °C; 3h	12, 220	(González-Fernández et al., 2012b)	
Scenedesmus	70, 80 °C; 25 min	9, 57	(González-Fernández et al., 2012a)	
Chlamydomonas sp., Scenedesmus sp. and Nannochloropsis sp.	55 °C; 12-24 h	Decrease	(Alzate et al., 2012)	
Acutodesmus obliquus and Oocystis	55 °C; 12-24 h	Decrease	(Alzate et al., 2012)	
Microspora sp.	55 °C; 12-24 h	4	(Alzate et al., 2012)	
Chlorella sp. and Scenedesmus sp.	50, 80 °C; 30 min	4, 14	(Cho et al., 2013)	
Thermochemical pretreatment				
Chlorella vulgaris	120 °C; pH 2; 20, 40	59.7, 64.7	(Mendez et al., 2013)	
Chlorella vulgaris	120 °C; pH 10; 20, 40	71.3, 73.2	(Mendez et al., 2013)	
Chlorella sp.	121 °C; 10 bar; 30	30	(Bohutskyi et al.,	
Nannochloropsis sp.	121 °C; 10 bar; 30 min, 21 g NaOH/ L	40	(Bohutskyi et al., 2014)	
Hydrothermal pretreatments				
Chlamydomonas sp., Scenedesmus sp. and Nannochloropsis sp.	110, 140 °C; 1-1.2 bar; 15 min	19, 33	(Alzate et al., 2012)	
Acutodesmus obliquus and Oocystis	110, 140 °C; 1-1.2 bar; 15 min	11, 31	(Alzate et al., 2012)	
Microspora sp.	110, 140 °C; 1-1.2 bar;	62, 50	(Alzate et al., 2012)	
Chlorella sp. and Scenedesmus sp.	15 min 140 °C; 30 min	20	(Cho et al., 2013)	
Chlorella vulgaris	120 °C; 30 min	90	(Mendez et al., 2014)	

# Table 2.1 continued

Species	Pretreatment conditions	Methane yield enhancement (%)	References	
Thermal pretreatment with				
Chlamydomonas sp., Scenedesmus sp. and Nannochloropsis sp.	170 °C; 6 bar; 15 min	46	(Alzate et al., 2012)	
Acutodesmus obliquus and Oocystis	170 °C; 6 bar; 15 min	55	(Alzate et al., 2012)	
Microspora sp.	170 °C; 6 bar; 15 min	41	(Alzate et al., 2012)	
Scenedesmus biomass	170 °C; 8 bar; 30 min	81	(Keymer et al., 2013)	
Chlorella vulgaris	170 °C; 20 min	85	(Mendez et al., 2014)	
<b>Microwave treatment</b> Nannochloropsis salina	2450 Hz; 5 times	40	(Schwede et al., 2011)	
<b>Ultrasound</b> Chlamydomonas sp., Scenedesmus sp. and Nannochloropsis sp.	10, 27, 40, 57 MJ/ kgTS	14, 14, 14, 12	(Alzate et al., 2012)	
Acutodesmus obliquus and Oocystis	10, 27, 40, 57 MJ/ kgTS	6, 8, 13, 13	(Alzate et al., 2012)	
Microspora sp.	10, 27, 40, 57 MJ/	23, 18, 18, 22	(Alzate et al., 2012)	
Scenedesmus biomass	kg15 100-130 MJ/ kgTS	75 - 90		
Chlorella sp. and Scenedesmus sp.	39, 117and 234 MJ/ kgTS	6, 10, 15	(Cho et al., 2013)	
Nannochloropsis salina	600 W, 2450 MHz	Decrease	(Schwede et al., 2011)	
<b>French press</b> Nannochloropsis salina	10 MPa, 2 passes	33	(Schwede et al., 2011)	

Species	Protein isolation method and operating conditions	and Results	
Spirulina platensis	Solubilisation in NaOH, followed by precipitation at isoelectric pH.	~68 % protein content in the protein isolate	(Chronakis et al., 2000)
Tetraselmis sp.	Cell disruption by bead mill followed by ion exchange chromatography and dialysis	64% proteins and 34 % sugars in the protein isolate	(Schwenzfeier et al., 2011)
Nannochloropsis	Solubilisation at pH 12 at 60 °C followed30 % yield, 40.5 %by precipitation at pH 3.4protein content		(Gerde et al., 2013)
Chlorella vulgaris	Cell disruption by high pressure homogenizer ( $2 \times 2.7$ kbar), solubilisation at pH 12 followed by recovery using isoelectric precipitation and tangential flow filtration	76 % protein extraction yield	(Ursu et al., 2014)
Nannochloropsis	Pulsed electric field and pH assisted extraction	Selective extraction of pure proteins	(Parniakov et al., 2015)
Spirulina platensis	n-hexane defatting followed by 3 times water wash at pH 8 and dialysis for 48 h	85 % protein-N extraction	(Devi et al., 1981)
Scenedesmus sp.	Flash hydrolysis at 280 °C, 10s residence time	66 % protein extraction yield	(Garcia- Moscoso et al., 2013)
Tetraselmis sp.	Low temperature hydrothermal treatment at 150 °C, 20 min holding time, followed by repeated (4 times) ethanol extraction	15% protein extraction yield	(Eboibi et al., 2015)

Table 2.2	Microalgal	protein	extraction	studies re	ported in	literature
	microuigui	protein	extraction	studies ie	ponea m	morature.

# CHAPTER 3

# OPTIMIZATION OF PROTEIN EXTRACTION FROM SPIRULINA PLATENSIS TO GENERATE A POTENTIAL CO-PRODUCT AND A BIOFUEL FEEDSTOCK WITH REDUCED NITROGEN CONTENT<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Parimi, N.S., Singh, M., Kastner, J.R., Das, K.C., Forsberg, L.S., Azadi, P. 2015 Frontiers in Energy Research, **3**.

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# Abstract

The current work reports protein extraction from Spirulina platensis cyanobacterial biomass in order to simultaneously generate a potential co-product and a biofuel feedstock with reduced nitrogen content. S. platensis cells were subjected to cell disruption by high pressure homogenization and subsequent protein isolation by solubilisation at alkaline pH followed by precipitation at acidic pH. Response surface methodology (RSM) was used to optimize the process parameters - pH, extraction (solubilisation/precipitation) time and biomass concentration for obtaining maximum protein yield. The optimized process conditions were found to be pH 11.38, solubilisation time of 35 min and biomass concentration of 3.6 % (w/w) solids for the solubilisation step, and pH 4.01 and precipitation time of 60 min for the precipitation step. At the optimized conditions, a high protein yield of 60.7 % (w/w) was obtained. The protein isolate (coproduct) had a higher protein content (80.6 % (w/w)), lower ash (1.9 % (w/w)) and mineral content and was enriched in essential amino acids, the nutritious  $\gamma$ -lenolenic acid and other highvalue unsaturated fatty acids compared to the original biomass. The residual biomass obtained after protein extraction had lower nitrogen content and higher total non-protein content than the original biomass. The loss of about 50 % of the total lipids from this fraction did not impact its composition significantly owing to the low lipid content of *S. platensis* (8.03 %).

**Keywords**: *Spirulina platensis*; protein isolate; high pressure homogenization; response surface methodology; residual biomass; biofuel feedstock

# **3.1 Introduction**

The concept of biorefinery which proposes the integration of biofuel production processes with the extraction of co-product(s) such as proteins, pigments and other high-value compounds is the path forward to improve the sustainability and economic feasibility of microalgal processing technologies. The high protein (and nitrogen) content of algal feedstock is a major limitation to whole biomass to biofuel conversion processes such as hydrothermal liquefaction (HTL) and anaerobic digestion (AD). High protein feedstocks result in high nitrogen content in the fuel produced from HTL and ammonia toxicity in AD (Chen et al., 2008; López Barreiro et al., 2013). Thus, nitrogen removal through protein extraction could potentially improve the feedstock composition for biofuel applications, while generating a useful co-product. Microalgal proteins are comparable to conventional protein sources such as soymeal and eggs, and hence find potential applications in human nutrition and animal feed (Becker, 2007; Spolaore et al., 2006).

Pre-treatments such as mechanical cell lysis, enzymatic, thermal and chemical treatments result in improved component extraction by complete or partial degradation of the microalgal cell wall, thus improving the accessibility of the intra-cellular components. High pressure homogenization and ultrasonication were reported to enhance microalgal protein solubilisation, the former being the most effective method (Gerde et al., 2013; Safi et al., 2014). Autoclaving was reported as an effective pretreatment to improve lipid extraction from microalgae (Prabakaran & Ravindran, 2011).

Protein solubility is pH dependent. Highly acidic and alkaline conditions enhance the solubility of algal proteins by inducing net charges on the amino acid residues (Damodaran, 1996). Proteins are least soluble at their isoelectric pH and precipitate out. Thus, solubilisation

under alkaline conditions followed by precipitation at isoelectric pH is a useful strategy for obtaining crude protein isolates. Several authors reported protein extraction from green algae and cyanobacteria using this method (Choi & Markakis, 1981; Chronakis et al., 2000; Gerde et al., 2013; Safi et al., 2014; Ursu et al., 2014). Other parameters that could impact protein solubility include extraction (solubilisation or precipitation) time, solvent/biomass ratio (biomass concentration) and temperature (Abas Wani et al., 2006). High temperature causes protein denaturing and also increases the energy input for the overall process (Goetz & Koehler, 2005). Hence, heat treatment is undesirable in protein isolation processes.

Process optimization and statistical analysis is necessary to maximize protein extraction and determine the independent and interaction effects of various process parameters on the extraction yields. Response surface methodology (RSM) is a popular statistical method for optimization of process parameters while conducting the least number of experiments (Firatligil-Durmus & Evranuz, 2010). Protein extraction process optimization using RSM for non-algal sources and *C.pyrenoidosa* (green algae) was reported previously (Ma et al., 2010; Quanhong & Caili, 2005; Wang & Zhang, 2012; Zhang et al., 2007b).

The current study dealt with process optimization for maximizing protein extraction from the cyanobacterium (blue-green alga) *S. platensis*, and the generation of a residual biomass with lower nitrogen content than the original biomass for potential applications as a biofuel feedstock in whole biomass conversion processes such as HTL and AD. Cyanobacteria differ significantly from green algae in cell wall structure and biochemical composition. Unlike the latter which have a recalcitrant cell wall comprising of cellulose and hemicellulose (Payne & Rippingale, 2000), cyanobacteria such as *Spirulina* and *Nostoc sp.* have a peptidoglycan based cell wall (Palinska & Krumbein, 2000). Moreover, they have a higher protein and lower lipid content (Becker, 2007). These differences necessitate the optimization of process parameters for the specific phylum. S. platensis was chosen in the current study for two reasons. First, it is an edible cyanobacterium and hence its protein isolate is expected to have a high nutritive value. Second, it has a very high protein content (Cohen, 1997) and hence the impact of protein isolation on the biochemical composition of the residual biomass would be very striking in this species compared to those with a lower protein content. Although some reports on extraction of proteins from S. platensis may be found in literature, major knowledge gaps on process optimization, component fractionation and product characterization remain (Chronakis et al., 2000; Devi et al., 1981; Safi et al., 2013b). The current work aimed at filling these gaps in order to understand the fate of various cell components as a result of the fractionation process and identify the bottlenecks in the process. Some of the parameters described in literature to characterize protein isolates such as protein content, amino acid composition, mineral composition and molecular weight range of the proteins were reported for the protein isolate obtained in this study (Chronakis et al., 2000; Gerde et al., 2013; Safi et al., 2013a). Such knowledge is very useful in assessing the sustainability, scalability and economic feasibility of the process.

## **3.2 Materials and Methods**

### 3.2.1 Microalgae

*Spirulina platensis* was obtained from Earthrise Nutritionals LLC (Calipatria, CA) in dry powder form and was stored in sealed, air tight plastic packages at room temperature prior to use. The dry powder was mixed with deionized (DI) water to form biomass slurry at the desired concentration (solids content).

#### 3.2.2 Protein isolation process

*S. platensis* biomass slurry prepared at the desired concentration was subjected to a protein isolation process (Figure S3.1) which involved pretreatment of the biomass and subsequent extraction of proteins by solubilisation at alkaline pH using 1 M NaOH followed by precipitation from the supernatant (obtained from the previous step) at acidic pH using either 1M HCl or 1M HCOOH. The solid-liquid separation after the solubilisation and the precipitation steps was achieved by centrifugation at 8670 g for 35 min. The pellet and the supernatant from the solubilisation step are henceforth referred to as alkali pellet and alkali supernatant respectively, and those from the precipitation step are referred to as acid pellet and acid supernatant respectively. The acid pellet was the protein isolate. The combined fraction of the alkali pellet and acid supernatant was the residual biomass.

# Selection of pretreatment

A 6 % slurry of *S. platensis* biomass was subjected to three different pretreatments namely autoclaving, ultrasonication and high-pressure homogenization. Autoclaving was carried out at 121 °C with 103.4 kPa (15 psi) for 30 min. Ultrasonication was carried out using a probe sonicator (Biologics, Inc., VA) at 20 % maximum power for 60 min. High pressure homogenization involved two passes through a high pressure homogenizer (Constant systems LTD, UK) at 103.4 MPa (15 kpsi). The samples were placed on ice bath during ultrasonication and high pressure homogenization, and a chiller was attached to the latter unit to minimize sample heating. The control experiment did not involve any pretreatment. Each of the pretreated and control samples was subjected to protein solubilisation at pH 11 for 60 min followed by solid-liquid separation. The treatments were compared based on protein recovery in the

supernatant fraction. The cells were observed visually under an optical microscope (400 times magnification).

# Optimization of experimental conditions

#### Solubility curve determination

A 6 % *S. platensis* biomass slurry was subjected to cell disruption by high pressure homogenization and separated into aliquots. The pH of each aliquot was adjusted to various values in the range of 2-13 (with a step size of 1 unit) using either 1 M NaOH or 1 M HCl and stirred for 30 min before subjecting to solid-liquid separation. A graph of pH versus protein recovery in the supernatant was plotted to obtain the solubility curve.

# Statistical optimization

The design of optimization experiments and the statistical analysis was carried out using SAS based JMP Pro (version 10) statistical software. A Box-Behnken design based on RSM was employed to optimize the process conditions affecting protein solubilisation and precipitation. The optimization range for pH for both the steps was chosen based on the solubility curve data. The range for solubilisation and precipitation times was 10-60 min. The 60 min maximum was chosen based on literature which reported that increasing the solubilisation time beyond 60 min did not result in a significant increase in the extracted proteins from pH 11 sonicated, non-defatted algae biomass (Gerde et al., 2013). The chosen range for biomass concentration was 2-10 % solids, a typical solids range of harvested algal biomass.

Based on the design, set of 15 and 10 experiments were carried out for the solubilisation and precipitation steps respectively (Tables S3.1 and S3.2). A second degree polynomial with the following general equation was fit to the data obtained from the solubilisation experiments:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3$$
(1)

where, Y was the protein recovery in the alkali supernatant,  $X_i$  (i=1,2,3) was the coded dimensionless value of an independent input variable  $x_i$ (i=1,2,3) in the range of -1 to 1. The independent input variables were  $x_1$ (pH),  $x_2$ (solubilisation time),  $x_3$ (biomass concentration).  $A_0$ was the constant term,  $A_i$  (i=1,2,3),  $A_{ii}$  (i=1,2,3) and  $A_{ij}$ (i=1,2,3; j=2,3; i≠j) are the linear, quadratic and interaction regression coefficients. The variables were coded according to the following equation:

$$X_i = (x_i - x_0) / \Delta x_i, i = 1, 2, 3$$
 (2)

where  $x_0$  was the real value of the center point of each input variable and  $\Delta x_i$  was the step change.

Protein precipitation from the alkali supernatant was carried out using 1 M HCOOH obtained at the RSM optimized conditions. A second degree polynomial with the following general equation was fit to the data obtained from the precipitation experiments:

$$Y = B_0 + B_1 X_1 + B_2 X_2 + B_{11} X_1^2 + B_{22} X_2^2 + B_{12} X_1 X_2$$
(3)

where, Y was the protein recovery in the acid pellet,  $X_i$  (i=1,2) was the coded dimensionless value of an independent input variable  $x_i(i=1,2)$  in the range of -1 to 1. The independent input variables were  $x_1(pH)$  and  $x_2(precipitation time)$ .  $B_0$  was the constant term,  $B_i$  (i=1,2),  $B_{ii}$  (i=1,2) and  $B_{ij}(i=1; j=2)$  were the linear, quadratic and interaction regression coefficients. The input variables were coded in a manner similar to the solubilisation step variables. The coefficient of determination  $(R^2)$  and the scattered plots between the experimental and predicted protein recoveries were obtained. The significance of the regression coefficients of the polynomial equations was determined using the student's t test and p value. Optimum process conditions were obtained from the response surface analysis and were experimentally validated.

## 3.2.3 Analytical methods

#### Total nitrogen, protein and amino acid analysis

A HACH high range total nitrogen assay method (HACH Corporation, Loveland, CO) was used to measure the total nitrogen concentration (mg L<sup>-1</sup>) in each sample. The nitrogen concentration obtained was multiplied by a factor of 6.25 to obtain the protein concentration (Chronakis et al., 2000; Piorreck et al., 1984; Safi et al., 2013a). A modified Lowry protein assay was used to determine the hydro-soluble protein content (Lowry et al., 1951). Bovine serum albumin (BSA) was used to prepare the standard curve for Lowry protein quantification. Nitrogen content (%N on dry basis) was obtained from the C, H, N, S elemental analysis carried out using a LECO brand analyzer (Model CHNS-932) according to the methods described in ASTM D 5291 and D 3176 (Jena et al., 2011a). Protein content (based on elemental analysis) was determined by multiplying the nitrogen content by the Conversion factor of 6.25. Amino acid analysis and quantification was carried out by the University of Missouri Agricultural Experiment Station (Columbia, MO). The proteins in the feed and product fractions were visualized under denatured conditions by SDS-PAGE using a Bio-Rad Miniprotean System<sup>TM</sup> with Any kD<sup>TM</sup> gels (Bio-Rad Laboratories, Hercules, CA, USA) (Gerde et al., 2013).

Total solids content was determined by drying the samples at 105 °C for 4 h in a conventional oven (Sluiter et al., 2008a). Lipids were extracted by Folch extraction method using chloroform/methanol mixture (2:1 ratio) (Folch et al., 1957), followed by centrifugation at 2600 g for 10 min. The chloroform soluble fractions were analyzed for fatty acids by preparing fatty acid methyl esters (FAMES) by methanolysis (1 M methanolic HCl, 80°C, 16h) and subjecting to GC-MS analysis using a non polar DB-1 capillary column equipped with mass selective detector following procedures as described (York et al., 1986). All extracts were first analyzed without any internal standard, allowing the use of behenic acid (C:22:0, 10 µg) as an appropriate internal standard. Hydroxy fatty acids were subjected to trimethylsilylation following methanolysis to facilitate GC separation; the response factors of common normal chain saturated and unsaturated fatty acid standards, and 2-hydroxy myristic acid standard were normalized relative to that of behenic acid. Ash content was determined after drying the samples in a conventional oven for 4 h and then incinerating them in a furnace at 575 °C for 3 h using a slightly modified version of the NREL procedure (Sluiter et al., 2008b). The rest of biomass which comprises predominantly of carbohydrates and small amounts of other cellular components may simply be considered as the carbohydrate fraction for convenience. Thus, the carbohydrate content was determined by the difference (Valdez et al., 2014).

# PG analysis

The product fractions were delipidated by the Folch lipid extraction method described in section 2.4.2 and then subjected to PG component analysis. In order to identify and quantify PG amino acids, a portion of the delipidated samples was hydrolyzed in 6 M HCl for 16 h at 105 °C followed by methanolysis for 4 h at 80 °C to yield methyl esters of amino acids, and finally

derivatized with heptafluorobutyric anhydride (HFBA), which yields the *N*-heptafluorobutyrate (and *O*- heptafluorobutyrate for Serine and Threonine) derivatives of the PG derived amino acids (Pons et al., 2003). The method was modified slightly wherein trans-esterification with isoamylalcohol was not performed and 2-amino adipic acid (25 µg) was used as internal standard. The resulting methyl esterified, HFBA derivatives were analyzed by GC-MS analysis using the DB-1 capillary column programed to 240 °C. For PG carbohydrate analysis, a separate aliquot was hydrolyzed in 1M HCl for 2 h at 105 °C followed by methanolysis for 6 h at 80 °C followed by N-acetylation (acetic anhydride/pyridine in methanol, 1:1:10 v/v, 45 min, 50 °C) and trimethylsilylation using "Tri-Sil" reagent (20 min, 80 °C) (York et al., 1986). Carbohydrates were measured relative to the internal standard myo-inositol (20 µg). The resulting HFBA-amino acids and TMS-methyl glycosides of monosaccharide sugars were analyzed separately by GC-MS analysis using a 30 meter DB-1 capillary column with electron impact mass fragmentation and detection, using temperature programs optimized for separately analyzing the amino acid and carbohydrate derivatives.

## **3.3 Results and Discussion**

# 3.3.1 Protein isolation optimization

# Comparison of different pretreatments

The results indicated that both high pressure homogenization and ultrasonication resulted in a higher protein recovery in the supernatant compared to control (Figure S3.2). High pressure homogenization was the better of the two pretreatments with a protein recovery of 83.5 % as opposed to 69.9 % in case of ultrasonication. Microscopic observation of the disrupted cells showed greater cell disruption with the former compared to the latter (Figure S3.3). Similar trend was reported for various algae and cyanobacteria (Safi et al., 2014; Ursu et al., 2014). Cell counting revealed that high pressure homogenization resulted in a near-complete cell lysis with disruption efficiency greater than 99 %, thus releasing most of the intra-cellular proteins. Autoclave treatment was the worst among all pretreatments with a protein recovery of only 29 %, which was slightly lower than the 32.1 % in the control. No visible cell disruption was observed under the microscope for the autoclaved *S. platensis* cells, explaining the lack of improvement in protein recovery. Thus, high pressure homogenizer based cell disruption was chosen as a pretreatment for all further protein isolation experiments.

#### Protein solubility curve

The solubility curve (Figure 3.1) showed that protein solubility (recovery in the supernatant) decreased with increasing pH in the acidic range of 2-4 and increased steadily in the range of 4-7. Least solubility was observed in the proximity of pH 4. High solubility (>75 % recovery) was observed in the alkaline range of 7-12. However, under extremely high alkaline conditions (beyond pH 12) the solubility decreased notably. This could be a result of significant protein denaturation and clustering, rendering the proteins insoluble (Haque et al., 2005). The variation in protein recovery was only about 10 % in the entire pH range of 6-12, although the trend was irregular. Highest recovery was obtained at pH 11 and closely followed by pH 8. These results differed from those reported for green algae. For *Chlorella vulgaris* the solubilisation after cell lysis was 19 % higher at pH 12 compared to pH 7 (Ursu et al., 2014). For *Nannochloropsis* species, protein solubilisation was reported to increase with increasing pH all the way until 13 (Gerde et al., 2013). Thus, pH 11 and 8 were further explored under different experimental conditions to determine the better of the two for protein solubilisation. A 3% *S*.

*platensis* biomass slurry subjected to cell disruption by high pressure homogenization and protein solubilisation resulted in 87.9 % protein recovery at pH 11 as opposed to 77.8 % at pH 8. Similarly, *S. platensis* biomass at nearly the same solids content but disrupted using ultrasonication resulted in 58.2 % protein recovery at pH 11 while only 38.7 % at pH 8. Thus, pH 11 was better than 8 for protein solubilisation.

# Optimization of protein isolation using RSM

pH ranges of 10.5-12 and 3-5 that were in the proximity (within 1 unit) of the points of highest and least solubility (reported above) were chosen for the design of protein solubilisation and precipitation optimization experiments respectively. Figure 3.2(A) shows the scattered plot between experimentally determined and RSM predicted protein recoveries in the alkali supernatant at different levels of the input variables. The experimental recovery varied from 64.87 to 95.6 % (data presented in Table S3.1). The regression coefficients of the second degree polynomial used to fit the protein recovery data, the standard error in their estimation and the statistical analysis are presented in Table 3.1. The regression equation obtained from the analysis was as follows:

$$Y = 93.03 - 1.54X_{1} + 1.32X_{2} - 10.36X_{3} - 1.72X_{1}^{2} - 2.33X_{2}^{2} - 9.16X_{3}^{2} - 0.55X_{1}X_{2} - 3.57X_{1}X_{3} + 1.95X_{2}X_{3}$$
(4)

The predicted recoveries were highly significant (p = 0.0027) and the coefficient of determination ( $\mathbb{R}^2$ ) for this model was 0.97, indicating a good fit. The results from the t test showed that biomass concentration was a highly significant factor (p=0.0001) in impacting protein recovery. The other two factors, pH and solubilisation time were not significant in the chosen range. However, the interaction of pH and biomass concentration was slightly significant

(p= 0.0522 < 0.1). Among the quadratic effects, only the quadratic biomass concentration term was highly significant (p=0.0015). The rest of the interaction and quadratic terms were not significant. The optimal values for pH, solubilisation time and biomass concentration determined by RSM were 11.38, 35.32 min and 3.61 % (w/w) solids respectively, and the predicted value of the response (protein recovery) at these conditions was 96 %.

Formic acid is a weak organic acid compared to hydrochloric acid which is a strong inorganic acid. In a comparative study, protein recovery in the acid pellet (protein isolate) was 71.7 % when precipitation was carried out using HCl and 71.5 % using HCOOH at the same experimental conditions. Thus, the substitution of HCOOH for HCl did not show any significant impact on protein precipitation. The former is more preferable than the latter when the residual biomass is intended to be used for biofuel production processes because chloride ions can corrode reactor vessels in thermochemical processes such as HTL (Kritzer, 2004), and the NaCl formed as a result of NaOH and HCl added during the protein isolation process can be toxic to the microbes in biochemical processes such as AD (Chen et al., 2008). Thus, HCOOH was used for protein precipitation in all further experiments.

Figure 3.2(B) shows the scattered plot between experimental and RSM predicted protein recoveries in the acid pellet at different levels of the input variables. The experimental recovery varied from 67 to 74.5 % (data presented in Table S3.2). The regression coefficients of the second degree polynomial used to fit the protein recovery data, the standard error in their estimation and their statistical analysis are presented in Table 3.2. The regression equation obtained from the analysis is as follows:

$$Y = 73.43 - 0.72X_1 + 1.28X_2 - 5.11X_1^2 + 1.49X_2^2 - 0.48X_1X_2$$
(5)

The predicted recoveries were significant (p = 0.01) and the coefficient of determination ( $R^2$ ) for this model was 0.95, indicating a reasonably good fit. The results from the t test showed that precipitation time was a significant factor (p=0.03) in impacting the protein recovery. The quadratic regression term for pH was highly significant (p=0.0014) but not the linear term, implying a quadratic dependence of protein recovery on pH in the chosen range. The quadratic term for precipitation time was slightly significant (p=0.0823<0.1). However, the interaction of pH and time was not significant implying both of these factors are independent of each other in the chosen range. The model predicted the solution to be a saddle point. However, based on single parameter profiles, the optimum conditions for maximum protein precipitation were determined as pH 4.01 and precipitation time of 60 min. The predicted value of the response (protein recovery) at these values was 76.2 %.

The RSM predicted maximum for overall protein yield after the alkali solubilisation and acid precipitation steps was calculated as 73.15 %. The experimentally determined protein recovery in the alkali supernatant and acid pellet at the RSM optimized process conditions for the solubilisation and precipitation steps were 86 % and 70.6% respectively. Although the experimental recoveries for the both the steps were lower than the theoretically predicted values, the variation (10.4 % and 7.3 % respectively) was within acceptable limits, considering the scale of operation (the amount of biomass used in each of the optimization experiments was 10 times lower than that used in the protein isolation process at the optimized conditions), handling and instrumental errors. The overall experimental protein yield at the optimum conditions was 60.7 %.

## 3.3.2 Component fractionation among the product fractions

Figure 3.3(A) shows the fractionation of various components between the protein isolate and the residual biomass obtained at the RSM optimized process conditions. The overall yield of total nitrogen and hence the yield of total protein in the protein isolate was 60.7 %. This value was higher than the yields reported in literature for proteins extracted using alkali-acid method from green algae (Gerde et al., 2013; Ursu et al., 2014) but lower than the 80 % yield reported for *S. platensis* protein isolates (Devi et al., 1981). The higher yield reported in the latter case was a result of the use of hexane defatted biomass as the starting material and the repeated (three times) aqueous extraction and dialysis steps. Lowry protein assay estimated that 56.9 % of soluble proteins were recovered in the protein isolate affecting a lower recovery in the residual biomass. The total solids fractionated almost equally between the two product fractions and so did the total lipids. However, carbohydrate recovery was higher in the residual biomass compared to the protein isolate.

The calculated purity or the protein content (% w/w) in the protein isolate was 80.6 %, which was 12.2 % higher than *S. platensis* biomass. This value of protein content was higher than that reported in literature for the protein isolate obtained from *S. platensis* using a slightly different procedure (Chronakis et al., 2000). Recovery of non-protein components in the protein isolate due to co-precipitation of insoluble carbohydrates, cell wall PG fragments (composed of amino sugars) and lipids limited the purity of this fraction. The PG fragments from the cell wall of *S. platensis* did not possibly degrade into their respective sugar and peptide components under the relatively mild pH (=4) condition used in the protein precipitation process resulting in their co-extraction with proteins (Vollmer, 2008). Further, the residual biomass fraction had an undesirably high nitrogen and protein content (7.6 % and 47.5 % respectively) indicating
incomplete protein extraction, the loss of non-protein components due to co-extraction with proteins and the presence of PG fragments. A PG composition analysis based on the diagnostic markers, diaminopimelic acid (DAP) and N-acetyl muramic acid (NAMA), revealed the presence of PG fragments in both the protein isolate and the residual biomass fractions. Although the latter had a slightly higher proportion of all PG components compared to the protein isolate, their overall contents were very low compared to other cellular components. The contribution of amino sugars towards the total nitrogen and carbohydrate content in both the fractions was also extremely low (0.16 % and 1.39 % of the total estimated nitrogen in the two fractions respectively). Thus, a further reduction in the nitrogen content of the residual biomass may be achieved only by repeated protein extractions involving additional processing steps and/ or other unit operations. However, such procedures would demand higher processing costs and other resources, and may negatively impact the scalability of the process. Hence, this idea was not investigated in this work.

#### 3.3.3 Initial biomass, protein isolate and residual biomass characterization

Figure 3.3(B) shows the nitrogen and protein (based on elemental analysis), lipid, carbohydrate and ash contents in the original biomass, the protein isolate and the residual biomass obtained at the RSM optimized conditions. The original *S. platensis* biomass comprised of 10.95 % nitrogen, 68.4 % total protein and 6.7 % ash by weight. Analysis of the protein isolate and the residual biomass revealed higher nitrogen and protein contents and lower lipid, carbohydrate and ash contents in the protein isolate compared to the residual biomass, which was in accordance with the desired outcome. The former was enriched in proteins while the latter was enriched in non-protein components. Although only 50 % of the total lipids were recovered in

the residual biomass, this did not have a huge impact on its composition due to the low lipid content of the original *S. platensis* biomass (8.03 %).

The PG carbohydrate analysis method described in section 3.2.3 also quantified non-PG originated sugars present in the biomass in addition to the PG amino sugars. The relative composition (% w/w) of the detected sugars in the protein isolate and the residual biomass fractions are shown in Figure 3.4(A). A major proportion of the sugars were glucose, which accounted for 77.50 % and 63.84 % of the total sugars (by weight) in each of these fractions respectively. This was expected, given that glucose is the most abundant sugar present in *S. platensis* (Shekharam et al., 1987). Galactose accounted for 8.24 % and 12.72 % in the protein isolate and the residual biomass respectively. The PG amino sugars NAMA and GlcNAc accounted for 6.11 % and 11.39 % of the total sugars respectively in the residual biomass. In the protein isolate the proportions of these amino sugars were 1.52 % NAMA and 5 % GlcNAc. Small amounts of mannose, 3-methyl hexose and fucose were also detected in both of these fractions.

Figure 3.4(B) shows the relative composition (% w/w) of the fatty acids detected by FAMES analysis in the protein isolate and the residual biomass. C16:0 (Palmitic acid) was the dominant fatty acid in both the fractions, as was the case for original *S. platensis* biomass (Cohen, 1997). However, this fatty acid represented 81.83 % of the total fatty acids in the residual biomass but only 46.76 % of the protein isolate. The latter contained significant amounts of mono and poly unsaturated fatty acids (C16-18) while the residual biomass had very small amounts. These and other fatty acids typically originate from membrane phospholipids where they are acylated to moieties carrying choline (phosphatidyl choline) and other polar head groups (Hoiczyk & Hansel, 2000). An  $\alpha$ -hydroxy fatty acid (2-OH-C17:0) was detected in low levels in

the protein isolate, but not in the residual biomass. These results clearly indicated that the protein isolate was enriched in poly unsaturated fatty acids while the residual biomass was enriched in saturated fatty acids. The former had a higher proportion of the essential fatty acid,  $\gamma$ -lenolenic acid (C18:3) compared to the original *S. platensis* biomass. This and other unsaturated fatty acids can be separated from the protein isolate using methods such as supercritical CO<sub>2</sub> extraction and urea complex formation (Cohen et al., 1993; Mendes et al., 2005) to yield high-value coproducts. The lower proportion of unsaturated fatty acids in the residual biomass is favorable for biofuel production processes because they could result in lower oxidative stability (rancidification) of the generated biofuel (Gunstone, 1967).

SDS-PAGE analysis revealed that several lighter (low protein concentration) bands observed in the molecular weight range of 25-100 kDa in original *S. platensis* biomass were not found in the disrupted biomass implying protein degradation as a result of cell disruption by high pressure homogenization (Figure S3.4). The bands around 100 kDa and 55 kDa were the most prominent ones among both the protein isolate and residual biomass fractions, although they were lighter in the latter indicating lower concentration of these proteins in this fraction. Thus, a higher proportion of the high molecular weight proteins fractionated into the protein isolate. Some of the bands observed in the disrupted biomass between 15 kDa and 20 kDa were not observed in the protein isolate and the residual biomass fractions suggesting that these low molecular weight proteins degraded into peptide components during the protein isolation process. Further, the small dark band at the bottom of the gel in the original biomass was observed only in the residual biomass and not in the protein isolate, indicating that the low molecular weight peptides and free amino acids typically present in algae remained in the residual biomass. The protein isolate obtained at a low ash content of 1.9 % was freeze dried and further analyzed for amino acid and mineral contents. The results presented in Table 3.3 show that the variation in the composition of a majority of the amino acids between the protein isolate and the edible original *S. platensis* biomass was low (below 10 %). The contents of 6 out of the 8 essential amino acids were slightly higher in the protein isolate. *S. platensis* biomass has widely been accepted as a rich protein source for humans and animals (Becker, 2004) and hence the protein isolate could potentially be used in these applications. The predominant minerals present in the protein isolate were aluminum, calcium, iron, potassium, magnesium, sodium, phosphorus, sulfur and silicon (Table S3.4). Except for sodium, the composition of all the elements was lower than original *S. platensis* biomass (Jena et al., 2011b) and hence is within agreeable limits for nutritional purposes. The excess sodium originated from the NaOH added during the solubilisation step.

# **3.4 Conclusion**

In this study, protein isolation from *S. platensis* cyanobacterium was carried out using the alkali-acid method after cell disruption using high pressure homogenization. The process conditions were optimized using RSM. At the optimized conditions, the proteins were extracted at a high yield of 60.7 % and content of 80.6 %. Further improvement of protein extraction was limited by co-fractionation of the non-protein components into the protein isolate and incomplete protein precipitation. The extracted protein isolate was enriched in proteins, essential amino acids and unsaturated fatty acids, and had a lower ash and mineral content compared to the original biomass. Such a composition is suitable for human food or animal feed applications. The residual biomass had a lower protein and nitrogen content than the original biomass and was

enriched in carbohydrates and saturated lipids, a composition better suited for biofuel applications such as HTL and AD.

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Source	Estimate	Std error	t ratio	<b>p</b> >   <b>t</b>
Intercept	93.03	1.6256	57.23	< 0.0001*
<b>X</b> <sub>1</sub>	-1.54	0.9955	-1.54	0.1834
<b>X</b> <sub>2</sub>	1.32	0.9955	1.32	0.2434
X <sub>3</sub>	-10.36	0.9955	-10.4	$0.0001^{*}$
$x_1x_2$	-0.55	1.4078	-0.39	0.7134
X <sub>1</sub> X <sub>3</sub>	-3.57	1.4078	-2.54	0.0522
X <sub>2</sub> X <sub>3</sub>	1.95	1.4078	1.38	0.2253
$x_1^{2}$	-1.72	1.4653	-1.17	0.2941
$x_2^2$	-2.33	1.4653	-1.59	0.1721
$x_{3}^{2}$	-9.16	1.4653	-6.25	0.0015*

**Table 3.1** Estimate of the regression coefficients for the alkali solubilisation optimization model

 and their statistical significance determined by student's t test.

\* Significant (p< 0.05)

	U		5	
Source	Estimate	Std error	t ratio	$\mathbf{p} >  \mathbf{t} $
Intercept	73.43	0.5908	124.28	< 0.0001*
<b>x</b> <sub>1</sub>	0.72	0.4036	1.78	0.1505
<b>X</b> <sub>2</sub>	1.28	0.4036	3.18	$0.0336^{*}$
$x_1x_2$	-0.48	0.4943	-0.96	0.391
$x_1^2$	-5.11	0.6472	-7.89	$0.0014^{*}$
$x_2^{2}$	1.49	0.6472	2.31	0.0823

**Table 3.2** Estimate of the regression coefficients for the acid precipitation optimization model

 and their statistical significance determined by student's t test

\* Significant (p< 0.05)

Table 3.3	Amino	acid	composition	(expressed	as	g/100	g	total	amino	acids)	of	the	original	S.

pl	atensis	biomass	and	the	protein	isolate
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Amino acid	Composition				
	Original biomass	Protein isolate			
Taurine	0.03	0.03			
Hydroxyproline	0.00	0.02			
Aspartic Acid	10.12	9.86			
Threonine*	4.92	4.85			
Serine	4.32	4.41			
Glutamic Acid	15.58	13.28			
Proline	3.66	3.79			
Lanthionine	0.00	0.00			
Glycine	5.06	5.24			
Alanine	7.48	7.31			
Cysteine	1.02	0.99			
Valine*	6.46	6.91			
Methionine*	2.38	2.40			
Isoleucine*	5.85	6.34			
Leucine*	8.91	9.80			
Tyrosine	4.40	5.07			
Phenylalanine*	4.71	5.16			
Hydroxylysine	0.16	0.15			
Ornithine	0.09	0.09			
Lysine*	4.84	4.49			
Histidine*	1.57	1.69			
Arginine	7.30	6.72			
Tryptophan*	1.14	1.42			

Essential amino acids



Figure 3.1. Solubility curve for *S. platensis* biomass. Error bars represent standard deviation of mean.



**Figure 3.2.** (**A**) Comparison between RSM predicted and experimental protein recovery for the alkali solubilisation step. The region within the dotted lines represents a 95 % confidence interval (**B**) Comparison between RSM predicted and experimental protein recovery for the acid precipitation step. The region within the dotted lines represents a 95% confidence interval



**Figure 3.3.** (**A**) Overall recoveries of various cell components in the protein isolate and the residual biomass fractions (**B**) The contents of various cell components in the original biomass, protein isolate and residual biomass. The analyzed product fractions were obtained at the RSM optimized conditions (Alkali step: (pH: 11.38, solubilisation time: 35.3 min, biomass concentration: 3.61 %); Acid step: (pH: 4.01, precipitation time: 60 min)). Error bars represent standard deviation of mean.



**Figure 3.4.** (A) Composition (% w/w) of each of the sugars present in the protein isolate and the residual biomass relative to total sugars in the respective fraction (B) Composition (% w/w) of each of the fatty acids present in the protein isolate and the residual biomass relative to total fatty acids in the respective fraction

# CHAPTER 4

# BIOMETHANE AND BIOCRUDE OIL PRODUCTION FROM PROTEIN EXTRACTED RESIDUAL SPIRULINA PLATENSIS<sup>1</sup>

<sup>1</sup> Parimi, N.S., Singh, M., Kastner, J.R., Das, K.C.2015. *Energy*, **93**, 697-704. Reprinted here with permission of the publisher.

# Abstract

The performance of the residual biomass obtained after protein extraction from the cyanobacterium *Spirulina platensis* as a feedstock for biomethane production via anaerobic digestion (AD), and biocrude oil production via hydrothermal liquefaction (HTL) pathways was investigated. The experimental methane yield and kinetic rate of methane production from protein extracted residual biomass (PERB) were higher by 30.4 % and 161 % respectively, compared to original *S. platensis* biomass (ORIB). The rate of methane production for PERB was also higher (by 38.9 %) than high pressure homogenizer disrupted biomass (DISB), although the yield was slightly lower (by 7.8 %). The lag phase time for methane production was the least for PERB among all the three substrates. On the other hand, HTL of PERB resulted in biocrude oil with slightly lower nitrogen content than ORIB (6.2 % and 7 % respectively), although at a reduced yield. A composition analysis using GC-MS revealed that the biocrude from PERB had a higher number of hydrocarbons and fatty acids and lower number of nitrogenous compounds compared to that from ORIB. A comparison of energy output and energy recovery in the AD and HTL processes suggested a better performance of PERB in the former process.

**Keywords:** *Spirulina platensis*; protein extraction; protein extracted residual biomass; anaerobic digestion; hydrothermal liquefaction

# Abbreviations

- AD Anaerobic digestion
- HTL Hydrothermal liquefaction
- PERB Protein extracted residual biomass
- **ORIB** Original biomass
- DISB High pressure homogenizer disrupted biomass
- BMP Biochemical methane potential
- ACP Aqueous co-product
- SR Solid residue
- DCM Dichloromethane
- HHV Higher heating value
- ER Energy recovery
- E<sub>output</sub> Energy output

# 4.1 Introduction

Algae (and cyanobacteria) are rich sources of protein for human and animal nutrition. However, extraction of algal proteins may not be an economically sustainable process by itself if the rest of the biomass is not used for any applications (Becker, 2007). Integration of the protein extraction process with biofuel production from the by-product of the process, the proteinextracted residual biomass (PERB), may improve the economics and sustainability of the overall process. PERB is expected to have a lower nitrogen and protein content, and a higher content of non-protein components such as carbohydrates and lipids compared to the original biomass (ORIB). Such a composition is favorable for biofuel production. However, since protein extraction is usually carried out on wet algae biomass, PERB is expected to have low solids content.

Anaerobic digestion (AD) and hydrothermal liquefaction (HTL) are wet processing technologies capable of converting biomass at low solids content to biofuels, thereby reducing the costs involved in extensive dewatering and drying. AD is a biochemical process which converts wet biomass into biogas using bacteria under anaerobic conditions. The biogas, mainly composed of methane and carbon dioxide, may be used directly as a fuel or purified and upgraded to biomethane which can be used in place of natural gas for several applications such as domestic fuel, electricity generation and transportation fuel (Bohutskyi & Bouwer, 2013). The AD process comprises of four stages namely hydrolysis, acidogenesis, acetogenesis and methanogensis. The first stage (hydrolysis) is rate limiting for AD of insoluble organic matter. Pretreatments such as mechanical cell disruption, thermal and chemical treatments can enhance AD by improving the accessibility of intracellular components by the hydrolytic enzymes produced by the anaerobic bacteria, thus improving the hydrolysis kinetics (Bohutskyi &

Bouwer, 2013; González-Fernández et al., 2012b; Mahdy et al., 2014; Schwede et al., 2011). Coproduct extraction as a pretreatment has also been reported to enhance algal AD, primarily due to the cell disruptive effect and higher solubilisation of organic matter. Ramos-Suárez and Carreras, 2014 investigated the anaerobic digestibility of residual Scenedesmus biomass obtained after enzymatic extraction of amino acids (Ramos-Suárez & Carreras, 2014). The amino acid extracted residues improved the yield and kinetic rate constant for methane production compared to the original Scenedesmus biomass. Astals et al., 2015 investigated anaerobic digestibility of Scenedesmus sp. after protein extraction using free nitrous acid pretreatment and found a 36% enhancement of methane production (Astals et al., 2015). Protein extraction to decrease the nitrogen content of algal/ cyanobacterial biomass may also help in reducing the formation of ammonia, a major inhibitory factor (Chen et al., 2008). McCarty, 1964 noted that ammonia inhibition in AD occurs at TAN (total ammonia nitrogen) concentrations of 1.5-3 g L<sup>-1</sup> at pH above 7.4 and a higher concentration of TAN becomes toxic irrespective of pH, leading to process failure (McCarty, 1964). A higher inhibitory range (1.7-14 g L<sup>-1</sup>) was reported by several authors and was said to depend on the nature of substrates and inocula, operating conditions and acclimatization periods (Chen et al., 2008). Free ammonia concentration of 1.1 g-N L<sup>-1</sup> was found to cause inhibition of batch AD of swine manure at pH 8 (Hansen et al., 1998). It was reported that AD of protein rich cyanobacterium Spirulina maxima containing upto 60 % protein content releases up to 7000 mg L<sup>-1</sup> of ammonia, a value in the inhibitory range (Samson & Leduyt, 1986). The presence of high amounts of ammonia in the biogas generated from algae also prevents its direct use as a fuel due to the possibility of harmful  $NO_x$  emissions, and adds to the costs involved in its purification to produce biomethane (Strik et al., 2006).

Hydrothermal liquefaction is a thermochemical process which converts wet biomass into biocrude oil in hot compressed water. This biocrude can be upgraded to generate liquid fuels for transportation and other applications (Dote et al., 1994). A major limitation of microalgal/cyanobacterial HTL is the presence of high protein content in the biomass, resulting in high nitrogen content in the final biocrude product (López Barreiro et al., 2013). This is highly undesirable as the combustion of this product would result in the formation of toxic oxides of nitrogen (NO<sub>x</sub>) which significantly impact the environment (Faeth et al., 2013). Moreover, the proteins which are hydrolyzed to amino acids at the hydrothermal conditions undergo further deamination, decarboxylation and cross reactions with carbohydrates and lipids to form complex nitrogenous compounds (Brown et al., 2010; Sato et al., 2004). This poses a difficulty in further upgrading the biocrude to fuel oil as it can lead to possible poisoning and deactivation of the catalysts and increased hydrogen demand during hydrodeoxygenation (Ross et al., 2010). Extraction of proteins from the microalgal/cyanobacterial biomass and the use of the residual biomass for HTL may result in the formation of biocrude oil with lower nitrogen content. Further, component extraction may help in reducing the formation of undesirable nitrogenous compounds by avoiding cross reactions. Cheng et.al., 2014 reported that the use of the residual biomass after lipid extraction from Nannochloropsis oceanica as a feedstock for HTL process prevented the formation of low grade, long chain amides and other nitrogenous compounds formed as a result of reaction between lipids and proteins (Cheng et al., 2014). Similar effect could be achieved by the use of protein extracted residual biomass as a HTL feedstock.

The current study investigated the performance of protein extracted residual *Spirulina platensis* biomass (PERB) as a feedstock for biomethane production via AD, in comparison to

non-protein extracted biomass. Although AD studies on protein/ amino acid extracted microalgal biomass were reported previously (Astals et al., 2015; Ramos-Suárez & Carreras, 2014), the type of biomass (organism) and the method of component extraction reported in the current study are significantly different. Green algae such as *Scenedesmus* differ from cyanobacteria such as *S. platensis* in cell wall structure and biochemical composition (Becker, 2007). Hence the impact of component extraction on the biodegradability and methane production is expected to be different among the two phyla. In addition, the method of component extraction may also impact the digestibility because mechanical, chemical or thermal pretreatments are usually applied during the extraction process. Thus the results presented in this work uniquely describe the impact of protein extraction (using the specific method) on AD for cyanobacteria.

The current study also investigated the performance of PERB as a feedstock for HTL process in comparison to original *S. platensis* biomass (ORIB). Such a study, describing the impact of protein extraction (using the specific method) on HTL process has not been reported for any microalgal/ cyanobacterial biomass previously to the best of our knowledge.

#### 4.2 Materials and Methods

# 4.2.1 Feedstock

Dry powder of *S. platensis* biomass was procured from Earthrise Nutritionals LLC (Calipatria, CA). Original biomass (ORIB) slurry at the desired total solids (TS) concentration was prepared by resuspending the dry *S. platensis* powder in deionized water. Disrupted biomass (DISB) was obtained by subjecting the ORIB slurry to cell disruption by high pressure homogenization (2 passes through a high pressure homogenizer (Constant systems LTD, UK) at

103.4 MPa (15 kpsi)). Protein extracted residual biomass (PERB) was generated by subjecting *S*. *platensis* biomass at 3.6 % solids content to cell disruption by high pressure homogenization and a subsequent protein isolation process involving two stages. The first stage involved protein solubilisation at pH 11.38 (adjusted using 1 M NaOH) for 35 min and subsequent centrifugation (8670 g, 35 min) in order to separate the pellet and the supernatant. The second stage involved protein precipitation from the supernatant (obtained from the first stage) at pH 4.01 (adjusted using 1 M HCOOH) for 25 min and subsequent centrifugation (8670 g, 35 min) to separate the pellet (protein isolate) and the supernatant. The pellet obtained from first stage was combined with the supernatant from the second stage to obtain PERB. The protein extraction experiments were carried out in duplicate. A part of the PERB obtained after protein extraction was dried in an oven at 50 °C and resuspended in deionized water to obtain biomass slurry at 15 % solids. This biomass slurry was used as feed to the HTL process.

#### 4.2.2 Biochemical methane potential

The biochemical methane potential (BMP) assay was used to evaluate the anaerobic digestibility of the substrates. BMP tests were conducted as batch experiments using the AMPTS II setup (Bioprocess Control, Sweden). Anaerobic sludge from a digester treating food waste was used as inoculum. ORIB, DISB and PERB were used as substrates. Each of the substrates and inoculum were premixed and loaded into the 500 mL bottle reactors after flushing with N<sub>2</sub> gas for 2-3 min to remove  $O_2$  from the headspace. The experiments were carried out at a working volume of 150 mL, substrate loading of 1 g volatile solids (VS) and substrate to inoculum ratio of 9:1 by volume. The inoculum to each BMP reactors had a COD of 2.41 g L<sup>-1</sup>, TS of 0.61 % and VS of 30 %. Each experiment was conducted in triplicate. Deionized water was used instead of substrate for control runs (carried out in duplicate) in order to determine the methane

productivity of the inoculum alone. The total organic load of the substrate/inoculum mixture in each reactor was 1.42 g COD except for control reactors (0.04 g COD). The temperature was maintained at 38 °C during the 30 day runs. The daily biomethane accumulation was recorded online. The actual daily cumulative methane production by each substrate was calculated by subtracting the recorded cumulative methane accumulation for the control from the corresponding value for the substrate-inoculum mixture on that particular day. The kinetic parameters for the BMP experiments were determined by fitting the cumulative methane production (mL  $g^{-1}VS$ ) to the modified Gompertz equation shown below (Gurung et al., 2012):

$$M = P \times \exp\left\{-\exp\left[\frac{R_m * e}{P}(\lambda - t) + 1\right]\right\}$$
(1)

where *M* is the cumulative methane production (mL g<sup>-1</sup>VS), *P* is the methane production potential (mL g<sup>-1</sup>VS),  $\lambda$  is the lag phase time (d) and  $R_m$  is the rate of methane production (mL g<sup>-1</sup>VS d<sup>-1</sup>).

# 4.2.3 Hydrothermal liquefaction and product separation

Hydrothermal liquefaction experiments were carried out in duplicate using PERB and ORIB at 15 % solids content as feedstock. The experiments were carried out in 100 mL batch reactors (Parr Instruments Co., Moline, PA) at a temperature of 350 °C and holding time of 30 min (Jena et al., 2011a). In a typical HTL run, the feed biomass slurry was transferred to the reactor which was then sealed and pressurized to about 3.45 MPa (500 psi) using helium gas. It was heated to 350 °C at a rate of 14 °C min<sup>-1</sup> using an electrical jacket and held at that temperature for 30 min. At the end of the run, the jacket was removed and the reactor was allowed to cool to ambient temperature. The ambient temperature and pressure were recorded, a fraction of the gas was sampled using Tedlar<sup>®</sup> sampling bags and the remaining gas was vented

out. The biocrude oil, aqueous co-product (ACP) and the solid residue (SR) in the product mixture were separated from each other using a combination of solvent (dichloromethane (DCM)) extraction, vacuum filtration, gravity separation and rotary evaporation (at 38 °C using a rotary evaporator) steps (Figure 4.1) (Eboibi et al., 2014). The biocrude oil obtained from PERB is henceforth referred to as PERB biocrude and that obtained from ORIB is referred to as ORIB biocrude. Similar notation is followed for the other HTL products.

#### 4.2.4 Analytical methods

C, H, N, S elemental analysis was carried out using a LECO brand analyzer (Model CHNS-932) (Jena et al., 2011a). Protein content was obtained by multiplying the N content with a factor of 6.25 (Chronakis et al., 2000; Piorreck et al., 1984; Safi et al., 2013). Total solids (TS) were determined by drying the samples in crucibles at 105 °C for 4 h in a conventional oven. After measuring the total solids, the ash content and volatile solids (VS) were determined by incinerating the crucibles in a furnace at 575 °C for 3 h using a slightly modified version of the NREL procedure (Sluiter et al., 2008). Elemental O content was obtained by the difference (based on C, H, N, S and ash contents). Chemical oxygen demand (COD) was analyzed by the HACH Reactor Digestion method (Method 8000) using HACH DRB 200 spectrophotometer (HACH Corporation, Loveland, CO) and digestion kit (Jena et al., 2011a).

The composition of the biocrudes and the ACPs was analyzed using a Hewlett-Packard (Model HP-6890) gas chromatograph in conjunction with a Hewlett-Packard (Model HP-5973) mass spectrometer with a mass selective detector. The dimensions of the GC column were: 30 m length, 0.25 mm internal diameter and 0.25  $\mu$ m film thickness. The method comprised of the following parameters: inlet temperature of 250 °C, detector temperature of 280 °C, flow at 1 mL min<sup>-1</sup> Helium, oven temperature at 40 °C held for 4 min followed by ramp up at the rate of 5 °C

min<sup>-1</sup> to 275 °C and held for 5 min. Sample size for injection was 1  $\mu$ L. A split ratio of 50:1 was used. The compounds were identified using Agilent Technologies Software (MSD Chemstation D.03.00.611) which carries out a probability-based matching of the unknown spectra to mass spectral library using National Institute of Standards and Technology's 2008 version. The ACPs were analyzed using high performance liquid chromatography (LC-20 AT, Shimadzu Corp., USA) equipped with a RID-10A refractive index detector and a 7.8 × 300 mm Corajel 64-H transgenomic analytical column for simple sugars and organic acids according to the method described by Jena et al., 2011 (Jena et al., 2011a). The gaseous products were analyzed by a gas chromatograph equipped with a thermal conductivity detector (Agilent 3000A micro-GC) to determine the concentrations of the gases (H<sub>2</sub>, N<sub>2</sub>, CO, CO<sub>2</sub>, CH<sub>4</sub> and other C2-C5). The columns used on the GC-TCD analyzer were: MS 5A PLOT (10 m length × 32 mm diameter), PLOT U (8 m × 0.32 mm), alumina PLOT (10 m × 0.32 mm), and OV-1 (10 m × 0.15 mm × 2.0 µm). The method was operated at the following parameters: sample size of 1 µL, oven temperature at 35 °C for 5 min, temperature ramp at the rate of 20 °C min<sup>-1</sup> to 200 °C and hold for 5.75 min.

#### *4.2.5 Theoretical calculations*

All recoveries and yields are reported on a percentage dry weight basis. Higher heating values (MJ kg<sup>-1</sup>) for the biocrudes and the feeds were calculated based on the elemental composition as described by Biller & Ross, 2011 (Biller & Ross, 2011). Energy output (MJ kg<sup>-1</sup>) and energy recovery (%) in the biocrudes and biomethane were calculated as follows (Anastasakis & Ross, 2015):

$$E_{output}(MJ kg^{-1}) = HHV_{product} \times Y_{product}$$
(2)

$$ER (\%) = \frac{HHV_{product}}{HHV_{feed}} \times Y_{product} \times 100$$
<sup>(3)</sup>

where  $Y_{product}$  is the product yield expressed as dry weight fraction (w/w).

Theoretical maximum yield of methane was calculated based on the elemental composition of the substrates according to the Buswell and Mueller, 1952 stoichiometric equation as modified by Richards et al., 1991 (Buswell & Mueller, 1952; Gallert et al., 1998; Richards et al., 1991):

$$C_{n}H_{a}O_{b}N_{c} + (n - 0.25a - 0.5b + 1.75c)H_{2}O$$

$$\rightarrow (0.5n + 0.125a - 0.25b - 0.375c)CH_{4}$$

$$+ (0.5n - 0.125a + 0.25b - 0.625c)CO_{2} + cNH_{4}^{+} + cHCO_{3}^{-}$$
(4)

### 4.3 Results and Discussion

#### 4.3.1 Feedstock composition

Table 4.1 shows the elemental composition, protein, ash and VS contents of ORIB and PERB. It was experimentally determined that there was not much variation in the composition of ORIB and DISB. Thus the theoretical parameters such as theoretical methane and HHV which were calculated based on these values were assumed to be the same for both these biomass. C, H, N, protein and VS content were lower, and ash content and C/N ratio were higher in PERB than ORIB.

# 4.3.2 Anaerobic digestibility and methane production

The cumulative methane production by the three substrates is shown in Figure 4.2(a). The experimental total methane production (yield) was the least for ORIB (181.1 mL  $g^{-1}VS$ ) owing to

the presence of intact cell wall which limits hydrolysis and access to the intracellular organic matter, resulting in low digestibility (González-Fernández et al., 2012a). This value is lower than the methane vields reported in literature for S. platensis (290-330 mL g<sup>-1</sup>VS) (Bohutskyi & Bouwer, 2013; Mussgnug et al., 2010), possibly due to the low inoculum input to the reactors in our study and inhibition of AD. Higher methane yields were obtained from DISB (254.5 mL g<sup>-</sup> <sup>1</sup>VS) and PERB (236.1 mL g<sup>-1</sup>VS). In both of these substrates, the S. platensis cells were subjected to cell disruption using high pressure homogenization (and additional protein extraction process in the case of PERB) which improved the access to intra cellular components and thus the digestibility. Enhancement of microalgal methane production by high pressure homogenization based cell disruption and protein/ amino acid extraction by various methods was reported previously (Astals et al., 2015; Ramos-Suárez & Carreras, 2014; Schwede et al., 2011). The total methane production (averaged for the two replicates) by the control after 30 days was only 14.4 mL. This was probably due to the low VS input (0.03 g) to the control reactors. However, on a VS basis the control exhibited a total methane productivity of 519 mL g<sup>-1</sup>VS. Several authors reported methane productivities from AD of various food wastes in the range of 298-482 mL g<sup>-1</sup>VS (Bouallagui et al., 2005; Cho et al., 1995; Zhang et al., 2007). Thus, the inoculum used in this study had a good methanogenic activity.

The calculated theoretical maximum and experimental methane yields for the three substrates are presented in Table 4.2. The theoretical yield of  $CH_4$  for original *S. platensis* reported in our work was comparable to the maximum theoretical yield reported for this species by Heaven et al., 2011, although our calculations were based on reaction stoichiometry and elemental composition (according to eq. 4), in contrast to Heaven et al., 2011 whose calculations were based on the empirical formulae and biochemical composition of proteins, lipids and

carbohydrates (Heaven et al., 2011). The experimental methane yield represented as percentage of theoretical is a clear indication of the extent of digestibility of a given substrate. Thus, the extent of digestibility was the highest for PERB and least for ORIB. The COD and VS consumed at the end of the BMP experimental runs were also the highest for PERB, supporting the inference that this substrate had a better digestibility than the other two (Figure 4.2(b)). The alkali treatment and acid hydrolysis steps involved in the generation of PERB must have resulted in the degradation of complex cellular organic compounds/ polymers into simpler, easily digestible compounds (for instance, complex carbohydrates into simple sugars) during the protein isolation process, resulting in higher digestibility and methane yield.

It can also be inferred from Figure 4.2(a) that the rate of methane production was much higher for PERB compared to the other two substrates. Table 4.3 shows the kinetic parameters for methane production obtained by curve fitting using the modified Gompertz equation for all three substrates. All the predicted parameter values were highly significant (p<0.001). The regression coefficients for curve fitting between the experimental and calculated methane production values for all the substrates was greater than 0.99 indicating a very good fit. Both the PERB and DISB substrates had a higher predicted methane production potential (BMP) and rate of methane production, and lower lag phase time than ORIB. Although the predicted BMP of PERB was lower than DISB (by 12.3 %), the rate of methane production was higher (by 38.9 %) and the lag phase much lower (by 6.3 days). This clearly indicated that the anaerobic bacteria carrying out the biochemical conversion could adapt to and digest the PERB substrate far more readily and at a faster rate compared to the other two substrates. This may be attributed to the presence of simpler organic compounds in PERB (as discussed above) which could easily be metabolized and converted by the anaerobic bacteria, and the possibility of lower ammonia

inhibition during AD of PERB. It has been reported that ammonia inhibition results in increased lag phase time and decrease in methane production rate (Peng et al., 2015; Van Velsen, 1979). Substrates with better kinetics and low lag phase times for methane production are highly favorable for scale up in industrial processes which operate in continuous mode as they can reduce the energy required for maintenance of the digesters, decrease the substrate residence times and improve the efficiency of the AD processes. Thus, PERB could potentially prove to be a much better substrate compared to the other two for commercial AD applications.

### 4.3.3 Hydrothermal liquefaction products

The biocrude oils obtained from PERB and ORIB were both dark brown in color and had a similar, strong odor. Figure 4.3(a) compares the yields of biocrude, aqueous soluble, solid and gaseous products obtained from PERB and ORIB. The yield of biocrude from ORIB was 26.9 % and from PERB was 13.7%, which was about half of the former. The lower yield from PERB was a result of its lower protein content because proteins contribute about 11-18 % of the total biocrude yield in HTL process (Biller & Ross, 2011). The decrease in biocrude yield was clearly reflected in the increase in gas and solid products yields from PERB. The increase in gas yield by about 21% may be attributed partly to gasification being better favored by PERB compared to ORIB, and partly to the breakdown of formic acid (added during the protein isolation process) present in PERB into CO and H<sub>2</sub> gases (Ross et al., 2010). The presence of a higher proportion of simpler, low molecular weight compounds in PERB formed as a result of disruption of cell structure and degradation of some of the cell components such as carbohydrates, proteins or lipids into simpler compounds during the protein isolation process may have favored gasification to liquefaction under the operated HTL conditions, also resulting in a reduction of biocrude yield. On the other hand, ORIB was not subjected to any treatments prior to the HTL process and

thus the slower hydrolysis and conversion kinetics may have favored liquefaction resulting in higher biocrude yields (López Barreiro et al., 2013). Several authors reported that algal HTL is influenced by the operating conditions (Eboibi et al., 2014; Jena et al., 2011a; López Barreiro et al., 2013). Thus, optimization of the HTL parameters for PERB may improve the biocrude yield by enhancing liquefaction. GC-TCD analysis of the gaseous products revealed that the relative composition (%) of CO and H<sub>2</sub> were higher in the PERB gas compared to the ORIB gas (2.2 % CO and 5 % H<sub>2</sub> in the former, and 0.3 % CO and negligible H<sub>2</sub> in the latter) confirming formate decomposition. CO<sub>2</sub> was slightly lower in the former compared to the latter (15.1 % and 16.1 % respectively). The higher SR yield from PERB compared to ORIB may be attributed to the higher ash content of PERB.

Figure 4.3(b) shows the nitrogen contents in ORIB and PERB, and the biocrudes, ACP and SR products obtained from them. ORIB biocrude had a nitrogen content of 7.0 % while PERB biocrude had 6.2 %. Thus, the nitrogen content in the HTL biocrude decreased by 0.8 % as a result of protein isolation. This decrease was not as high as desired and may be attributed to the undesirably high nitrogen content (7.6%) still remaining in PERB, which was the feed to the HTL process. The review article by Barreiro et al., 2013 highlighted that higher nitrogen content in the feed is reflected as higher nitrogen content in the biocrude and vice versa (López Barreiro et al., 2013). The nitrogen content of the SR also showed a similar trend. Thus, additional unit operations or alternative methods to further decrease the nitrogen content in PERB may result in obtaining biocrude with lower nitrogen content.

Figure 4.4 shows the cumulative chromatographic peak areas of different classes of organic compounds present in ORIB biocrude and PERB biocrude (further information on the compounds present in the biocrudes may be found in Table S4.1 and Table S4.2). PERB

biocrude comprised of a higher number of long chain aliphatic hydrocarbons (C10-C20) such as heptadecane and pentadecane and fatty acids such as n-hexadecanoic acid compared to ORIB biocrude. ORIB biocrude comprised of a number of long chain aliphatic amides such as hexadecanamide, N-methyl dodecanamide etc., which were not present in PERB biocrude. Such compounds have been identified in the biocrude obtained from *Spirulina* biomass previously and are formed by reactions between protein and lipid hydrolysates (Biller & Ross, 2011; Cheng et al., 2014). In addition, both the biocrudes contained a number of heterocyclic nitrogenous compounds such as derivatives of pyrrolidine and piperidine. The presence of such heterocyclic nitrogenous compounds in algal biocrude was also reported previously (Biller & Ross, 2011). These compounds are formed as a result of repolymerization of the decarboxylated and deaminated amino acid hydrolysates or their recombination with carbohydrate hydrolysates through Maillard reactions (López Barreiro et al., 2013; Toor et al., 2011). It may be inferred from the cumulative peak areas that PERB biocrude had a higher amount of long chain aliphatic hydrocarbons and fatty acids compared to ORIB biocrude. Although the cumulative peak area for nitrogenated heterocyclic compounds was higher for PERB biocrude, the cumulative peak area of all nitrogenous compounds (nitrogenated heterocyclic compounds, long chain aliphatic amides and other nitrogenous compounds) was higher for ORIB biocrude.

The yield of PERB ACP was lower than ORIB ACP. Both ACPs were light brown in color and had pH around 9. The COD was high, implying the presence of high amounts of organics. Both the ACPs were enriched in oxygen (about 90%) and had low nitrogen content (around 0.5 %). GC-MS analysis showed that PERB-ACP comprised primarily of nitrogenous compounds (several aliphatic straight chain, branched and heterocyclic amines) and ethanol. HPLC analysis of this aqueous co-product showed the presence of a few organic acids and

sugars - formate (3 mg mL<sup>-1</sup>), acetate (2 mg mL<sup>-1</sup>), lactate (2 mg mL<sup>-1</sup>), ethanol (0.8 mg mL<sup>-1</sup>) and succinate (0.6 mg mL<sup>-1</sup>). Analysis of the mineral composition revealed that the content of most of the mineral elements present in significant amounts was higher in PERB ACP than in ORIB ACP (Table S4.3). The PERB biomass generation process resulted in the concentration of the mineral elements, as was evident from the higher ash content (about 3 times) present in this biomass compared to ORIB. Most of these mineral elements may have originated from original *S. platensis* biomass, except sodium which was added during the protein extraction process. The growth limiting nutrients of phosphorus and nitrogen were present in excess in both the ACPs compared to standard algal growth media (Xin et al., 2010). Thus, both the ACPs may potentially be used for algal cultivation. Such an application has previously been explored and validated using ACPs obtained from *S. platensis* and other microalgae (Biller et al., 2012; Jena et al., 2011b).

#### 4.3.4 Comparison of AD and HTL

Table 4.4 presents the elemental composition and HHV (energy content) of the feedstocks and the biomethane and biocrude products obtained from them, and the energy output and energy recovery in the AD and HTL processes. The HHV of PERB was lower than ORIB and DISB by 34 %. Yet, the energy recovery in the biomethane produced from PERB was higher than that from ORIB and DISB by 68.4 % and 19.8 % respectively. In terms of energy output from AD, PERB performed better than ORIB (by 11.3 %) but worse than DISB (by 20.7 %). The lower energy output from PERB compared to DISB was a result of lower biomethane yield.

The HHV of PERB biocrude was slightly lower than ORIB biocrude (by 8.3 %). Both these HHV values were about 72-78 % of the HHV of petroleum crude oil (42 MJ kg<sup>-1</sup>) (Matar & Hatch, 2001). The energy recovery in PERB biocrude was 29.9 % lower than ORIB biocrude.

This was clearly a result of lower biocrude yield from PERB. A comparison of energy output and energy recovery in the biofuel products (biomethane and biocrude) obtained from PERB and ORIB showed that AD performed better than HTL for the former biomass and vice-versa for the latter.

# 4.4. Conclusion

This study showed that biofuel production after protein extraction has some advantages over direct biofuel production from original *S. platensis* biomass. AD tests revealed that the protein extracted residual biomass was a far more biodegradable substrate with a higher kinetic rate of methane production and capable of producing higher amount of methane gas compared to the original biomass. HTL of PERB resulted in a biocrude with slightly lower nitrogen content and higher number of long chain aliphatic hydrocarbons and fatty acids compared to ORIB. Thus, the use of PERB proved to be beneficial to both the AD and HTL processes, although AD performed better in terms of product yield, energy output and energy recovery. Further optimization of the protein extraction and biofuel production processes may improve the yield of biomethane and biocrude oil that could be generated from PERB in order to better impact the economics and sustainability of the integrated process.

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residual biomass (PERB). All values are reported as weight percentages on a dry basis<sup>a</sup>.

 Parameter
 ORIB
 PERB

Table 4.1 Composition of the original S. platensis biomass (ORIB) and the protein extracted

C (%)	50.1(2.0)	35.3 (2.5)
H (%)	6.4 (0.3)	5.5 (0.2)
N (%)	11.0 (0.1)	7.6 (0.1)
O (%)	25.9 (1.4)	31.3 (3.2)
Ash (%)	6.7 (0.5)	20.3 (0.1)
VS (%)	93.3 ( 0.5)	79.7 (0.1)
C/N ratio	4.58 (0.1)	4.64 (0.3)
Protein content (%)	68.8 (1.9)	47.5 (0.8)

<sup>a</sup> Values in parentheses are standard deviations of means

SubstrateTheoretical CH4<br/>yield (mL g<sup>-1</sup>VS)Experimental CH4<br/>yield (mL g<sup>-1</sup>VS)CH4 yield as %<br/>of theoreticalORIB558.4181.1 (11.8)32.4DISB558.4254.5 (16.0)45.6

236.1 (13.1)

63.1

**Table 4.2** Theoretical and experimental  $CH_4$  yields for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)<sup>a</sup>.

<sup>a</sup>Values in parentheses are standard deviations of means

374.0

PERB

Substrate	$P(mL g^{-1}VS)$	$\mathbf{R}_{\mathbf{m}} \left(\mathbf{m}\mathbf{L} \mathbf{g}^{-1} \mathbf{V} \mathbf{S} \mathbf{d}^{-1}\right)$	λ (d)	$\mathbf{R}^2$
ORIB	213.4 (8.9)	15.6 (1.4)	14.9 (0.2)	0.9938
DISB	262.4 (2.4)	29.3 (1.1)	10.4 (0.1)	0.9979
PERB	230.0 (1.7)	40.7 (2.3)	4.2 (0.2)	0.9942

**Table 4.3** Methane production kinetic parameters for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)<sup>a</sup>

<sup>a</sup>Values in parentheses are standard errors

Sample	Elemental analysis (% w/w) <sup>a</sup>			HHV	Yield	Eoutput	ER (%)	
	Ν	С	Н	0	- (MJ kg <sup>-1</sup> )	(% w/w)	(MJ kg <sup>-1</sup> )	
AD								
Biomass								
ORIB	11	50.1	6.4	25.9	21.5			
DISB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			
Biomethane								
ORIB					55.5	11.2	6.2	28.8
DISB					55.5	15.7	8.7	40.5
PERB					55.5	12.4	6.9	48.5
HTL								
Biomass								
ORIB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			
Biocrude								
ORIB	7	68.6	8.7	15.8	32.7	26.9	8.8	41.1
PERB	6.2	64.3	8.5	21.1	30.0	13.7	4.1	28.8

**Table 4.4** Elemental composition and HHV of the feedstocks and biofuel products, and the energy output ( $E_{output}$ ) and energy recovery (ER) in AD and HTL processes.

<sup>a</sup>S (% w/w) detected in all the samples was found to be very low



Figure 4.1. Hydrothermal liquefaction and product separation process. SR - Solid residue; ACP

- Aqueous co-product.



Figure 4.2. AD of original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB). (a) Methane production per gram VS (input). (b) VS and COD consumed.



Figure 4.3. HTL of original biomass (ORIB) and protein extracted residual biomass (PERB).
(a) Products yield (b) Nitrogen content of products and feedstock. SR – Solid residue; ACP – Aqueous co-product.



**Figure 4.4.** GC-MS characterization of the biocrude from original biomass (ORIB biocrude) and the biocrude from protein extracted residual biomass (PERB biocrude). LHC – Long chain hydrocarbons (C10 - C20); AHC – Aromatic hydrocarbons; NHCC – Nitrogenated heterocyclic compounds; LAA – Long chain aliphatic amides; ANC – Other nitrogenous compounds; Ket – Ketones; Phe – Phenolics; FA – Fatty acids; Oxy – Oxygenates.

# CHAPTER 5

# EVALUATION OF MICROALGAL BIOMASS RESIDUES FROM PROTEIN FRACTIONATION PROCESSES AS SUBSTRATES FOR ANAEROBIC DIGESTION $^{\rm 1}$

<sup>&</sup>lt;sup>1</sup> Parimi, N.S., Singh, M., Kastner, J.R., Das, K.C. 2016. To be submitted to *Bioresource Technology* 

# Abstract

The methane yield and production efficiency (percentage ratio of experimental to theoretical yield), kinetic parameters, and energy recovery from the residues generated after protein fractionation using two different methods (high pressure alkali-acid (HPAA) method and low temperature hydrothermal (LTHT) method) were determined, and compared against the values obtained for the respective non-fractionated (untreated and cell disrupted) biomass substrates for three different microalgal species (Chlorella pyrenoidosa, Tetraselmis chuii and *Phaeodactylum tricornutum*). The methane yield (253.3-265.4 mL/gVS), production efficiency (60.8-90.2 %), kinetic rate of methane production (7.6-9.3 mL/gVS/d) and energy recovery (48.4-73.7 %) for the residues generated by the HPAA method were higher than the nonfractionated substrates. Among the residues obtained from LTHT method, only Chlorella pyrenoidosa (CP) residue resulted in a higher methane yield compared to both its nonfractionated substrates. Its methane production efficiency, rate and energy recovery were higher (by 27.1 %, 31.3 % and 30.2 % respectively) than the untreated but lower (by 11.6 %, 25.9 % and 9.4 % respectively) than the cell disrupted biomass. Across all species, the residues from HPAA method resulted in higher methane yields, rates of methane production and energy recoveries than those from LTHT method. A preliminary characterization of the protein isolates (co-products) obtained from HPAA process suggested a potential for food or feed applications.

Keywords: Microalgae; methane yield; protein fractionation; residues; protein isolate

# Abbreviations

- AD Anaerobic digestion
- CP Chlorella pyrenoidosa
- TC Tetraselmis chuii
- PT Phaeodactylum tricornutum
- HPH High pressure homogenizer based cell disruption
- HPAA High pressure alkali-acid method
- LTHT Low temperature hydrothermal treatment method
- U Untreated biomass
- D Cell disrupted biomass
- R1 Residues from HPAA method
- R2 Residues from LTHT method
- HHV Higher heating value
- Eoutput Energy output
- ER Energy Recovery
- Prt Protein isolate from HPAA fractionation method
- P Methane production potential (mL/gVS)
- $R_m$  Rate of methane production (mL/gVS/d)
- $\lambda$  Lag phase time (d)

Microalgal anaerobic digestion (AD) has been receiving increasing attention in the recent past owing to its ability to process wet algal biomass (and thereby eliminating the need for expensive dewatering processes), the low energy input to the digesters and the ability to convert all organic compounds (carbohydrates, lipids and proteins) to biogas (Passos et al., 2014). However, cell wall recalcitrance and the low C/N ratio owing to the high protein content of microalgal feedstock are major limitations to AD (Chen et al., 2008; Samson & LeDuy, 1983; Yen & Brune, 2007). Cell wall recalcitrance is overcome by pretreatments that cause complete or partial disintegration/ fragmentation of cell walls (Sialve et al., 2009). Co-digestion with carbon rich substrates could improve the C/N ratio and reduce ammonia inhibition (González-Fernández et al., 2012a). However, this approach is limited by the availability of co-digestion substrates with similar degradation kinetics (in order to maintain the C/N ratio) throughout the digestion process (Ward, 2015). An alternate approach to overcome the limitations of microalgal AD is based on protein/nitrogen fractionation and use of the residual biomass as feedstock. This integrated approach could benefit the overall process by enhancing AD (owing to cell rupture, hydrolysis of complex organic matter and decreased ammonia inhibition due to improved C/N ratio), and simultaneously generating a protein-rich co-product fraction (Ramos-Suárez et al., 2014). The high nutritive value of microalgal proteins is well documented in literature (Becker, 2007; Spolaore et al., 2006; Yaakob et al., 2014), implying the possible use of the protein fraction as food or feed supplement. Other potential applications of protein/ nitrogen extracts include nutrient recycle, fertilizer, feed to industrial fermentation processes and utility as biopolymer blends (Romero García et al., 2012; Zeller et al., 2013).

Protein extraction based on pH shifting method using alkali and acid generates residues that have lower nitrogen content, higher C/N ratio and an enriched non-protein composition (carbohydrates and lipids) relative to whole microalgal biomass (Cavonius et al., 2015; Parimi et al., 2015b; Ursu et al., 2014), hence are attractive substrates for AD. An alternative method to achieve microalgal cell rupture and generate residues with lower nitrogen content than the feed biomass that has been suggested in literature is based on low temperature (100-225 °C) hydrothermal treatment (LTHT) (Costanzo et al., 2015; Eboibi et al., 2015; Jazrawi et al., 2015). Proteins are hydrolyzed and fractionated into the aqueous phase, and solid residues with improved C/N ratios can be obtained at appropriate pretreatment temperatures. Such residues could have a positive impact on AD. The literature on AD of protein extracted microalgal residues is very limited to studies on Scenedesmus sp. (Astals et al., 2015; Ramos-Suárez & Carreras, 2014) and our previous work on the cyanobacterium Spirulina platensis. It is a known fact that microalgal digestibility is strain specific and is impacted by characteristics such as cell wall structure and biochemical composition (Bohutskyi & Bouwer, 2013; Sialve et al., 2009). Thus, a comparative study across multiple species, preferably with different cell characteristics, would provide more information on the benefits of utilizing protein extracted residues for biomethane production.

In this study, methane production from residues obtained after protein fractionation by HPAA method and LTHT method from 3 different microalgae was investigated. To the authors' knowledge this is the first time that the residues obtained by hydrothermal protein fractionation method were studied as substrates for AD. The species studied in this work included a freshwater green alga *Chlorella pyrenoidosa* (CP), a marine green alga *Tetraselmis chuii* (TC) and a marine diatom *Phaeodactylum tricornutum* (PT). *Chlorella* has a recalcitrant fibrillar polysaccharide (cellulose/hemicellulose) based cell wall, *Tetraselmis* has a more fragile glycoprotein based cell wall and the fusiform *Phaeodactylum tricornutum* has a cell wall composed of lipids and polysaccharides (such as glucomannans and frustulins) (Domozych, 1984; Takeda, 1991; Tesson et al., 2009). The biochemical composition of the species also differs widely. Additionally, this study also reports a preliminary characterization of the co-product fraction generated from the most beneficial fractionation process to prospect applicability for nutritional purposes.

#### **5.2 Materials and methods**

#### 5.2.1 AD substrates

*Chlorella pyrenoidosa* (CP) biomass was purchased from Starwest Botanicals, Inc. (Sacramento, CA). *Tetraselmis chuii* (TC) and *Phaeodactylum tricornutum* (PT) were purchased from Fitoplancton Marino (Spain). The algal biomass was reconstituted to the required total solids content using deionized water. The untreated biomass is denoted by U in all contexts of this manuscript.

Biomass U at 4.5 % solids were subjected to mechanical cell disruption using a high pressure homogenizer (Constant systems LTD, UK) to obtain cell disrupted biomass, D. The operating pressure and number of passes were 206.8 MPa and 3 for CP, 103.4 MPa and 1 for TC, and 103.4 MPa and 2 for PT, respectively. Throughout the cell disruption process, the high pressure homogenizer was cooled using a chiller and the samples were collected on an ice bath. Cell lysis was observed under an optical microscope at 400x magnification (microscope images of intact and broken cells may be found in supplementary Figure S5.1).

Protein fractionation by HPAA method was carried out as described in our previous work (Parimi et al., 2015b). In brief, the method involved cell disruption of microalgal biomass (as described above) and a subsequent protein extraction involving the following steps: (i) solubilisation at alkaline pH 11.3 (adjusted using 1M NaOH) for 35 min; (ii) centrifugation at 7000 RPM for 25 min to separate the pellet from the supernatant; (iii) precipitation of proteins from the supernatant from step (ii) at acidic pH 4 (adjusted using 1M HCOOH) for 60 min; and (iv) centrifugation at 7000 RPM for 25 min to separate the pellet from the supernatant. The pellet and supernatant from steps (ii) and (iv) respectively were combined together to obtain the protein extracted residue, R1.

LTHT treatment was carried out in 75 mL batch reactors (Parr 5000 Multi Reactor System). Each of the reactor vessels was loaded with 7 g microalgal biomass (CP, TC or PT) and 40 g of DI water. The vessels were charged to a pressure of 300 psi (2.1 MPa) using Helium gas and sealed. A constant stirring rate of 300 RPM was maintained using a PTFE magnetic stir bar to avoid temperature gradients. The vessels were heated to 200 °C in the aluminum heating wells (using in-built band heaters) at a rate of ~10 °C/ min, and held at that temperature for 15 min. After the holding time of 15 min was elapsed, the reactors were removed from the heating wells and placed on an ice bath to cool down. The treated biomass was collected after depressurizing the reactors at ambient temperature and subjected to centrifugation at 8000 RPM for 20 min. The aqueous phase was decanted off. The solid fraction was resuspended in deionized water to obtain the protein extracted residue, R2.

## 5.2.2 Anaerobic digestion

Anaerobic digestion was carried out in batch mode in 120 mL glass bottles placed in a shaker incubator. The bottles were tightly sealed with rubber seals and aluminum caps to make

them air tight. The substrates comprised of non-fractionated (untreated and cell disrupted), and fractionated (by HPAA and LTHT methods) CP, TC and PT microalgal biomass. pH of all substrates was adjusted to 7 prior to digestion. The inoculum comprised of anaerobic sludge from a digester treating raceway grown microalgal biomass at mesophilic conditions. The total working volume and substrate loading were 60 mL and 0.3 gVS for CP and TC substrates, and 50 mL and 0.25 gVS for PT substrates respectively, and inoculum/substrate ratio was 1:2. The lower VS of inoculum relative to substrate was used to better understand the performance of various substrates without neutralization of any inhibition effects by the inoculum. Each of the bottles containing the substrates was flushed with nitrogen gas before inoculating to remove oxygen from the headspace. Control experiments were carried out using deionized water in place of substrate. Temperature was maintained at 35 °C and the agitation speed was 40 RPM. All experiments were carried out in triplicate for a total duration of 74 days. Methane produced from the inoculum alone (control reactors) was subtracted from the methane produced from the substrate-inoculum mixture. The kinetic parameters for the AD experiments were determined according to the modified Gompertz equation (Gurung et al., 2012).

#### 5.2.3 Analytical methods and theoretical calculations

Total solids (TS), volatile solids (VS) and ash content were measured according to standard methods (Sluiter et al., 2008a; Sluiter et al., 2008b). Total COD (tCOD) and soluble COD (sCOD) were measured by HACH reactor digestion assay (method 8000) using a DRB 200 spectrophotometer (HACH Corporation, Loveland, CO) and digestion kit. Soluble fractions for sCOD analysis were obtained by collecting the supernatant after centrifugation at 4700 RPM for 10 min. COD solubility (%) was estimated as the percentage ratio of sCOD to tCOD (100\*[sCOD/tCOD]). CHNS elemental analysis was carried out by Thermo-Scientific Flash

2000 elemental analyzer. Protein content was correlated to nitrogen content using the conversion factor of 6.25 (Piorreck et al., 1984; Safi et al., 2013). Biogas production was measured by volume displacement in a eudiometer water column (Selutec, Germany). Biogas was drawn from the headspace of the AD bottle using an airtight syringe for methane content analysis. Gas samples were analyzed for methane content using a gas chromatograph equipped with a flame ionization detector (Model SRI 310, SRI Instruments, Torrance, CA). This GC had a stainless steel column (80/100 HayeSep, 6" by 1/8"), oven temperature of 40 °C and detector temperature of 380 °C. The carrier gas was Helium (10 mL/min), fuel gas was Hydrogen (25 mL/min) and oxidizing gas was air (250 mL/min). The sample size of the biogas injected was 100 μL. Methane production was determined based on the biogas production and methane content. Experimental methane yield was the cumulative methane produced by the end of the digestion period per gram of volatile solids input. Amino acid analysis was carried out at the molecular structural facility at University of California, Davis (Davis, CA).

Theoretical maximum methane yields and higher heating values (HHV) were estimated from the elemental composition of the biomass as described in literature (Biller & Ross, 2011; Buswell & Mueller, 1952; Richards et al., 1991). Methane production efficiency was calculated as the percentage ratio of experimental methane yield to the theoretical maximum methane yield. Energy output ( $E_{output}$ ) and energy recovery (ER) were calculated based on experimental methane yield and HHV of feedstock and methane (55.5 MJ/ kg) using the following equations (Anastasakis & Ross, 2015):

$$E_{output}(MJ/kg) = HHV_{product} \times Y_{product}$$
(1)

$$ER (\%) = \frac{HHV_{product}}{HHV_{feed}} \times Y_{product} \times 100$$
<sup>(2)</sup>

#### 5.2.3 Statistical Analysis

Statistical analysis was carried out using JMP-Pro version 10 (SAS-based) software. Two-way Analysis of variance (ANOVA) was carried out to compare the treatment effects and determine the significance of the experimental results at p < 0.005.

# 5.3 Results and discussion

#### 5.3.1 Substrate characterization

Table 5.1 reports the results from the biochemical characterization of all the substrates used in this study. Cell disrupted biomass (D) was used as one of the substrates to differentiate the effects of cell disruption alone from protein fractionation, because fractionation methods usually involve or result in cell disruption. It was assumed that mechanical cell disruption by high pressure homogenization does not result in a significant change in composition and hence the ash, VS, COD and elemental (CHNSO) composition are reported to be the same for both U and D substrates. The freshwater microalga CP had a higher VS and lower ash content compared to the marine species (TC and PT). VS content was the highest for R2 biomass and least for R1 biomass across all the 3 species. This indicated that the LTHT process resulted in loss of minerals to the aqueous fraction reducing the ash content and increasing the VS content. On the other hand, the concentration of minerals in the biomass residues (R1) along with salt accumulation from the addition of alkali and acid during the HPAA process resulted in a higher ash and lower VS content.

Among the untreated biomass, CP-U had the highest nitrogen and protein content (9.3% and 58.1% respectively). In general, R1 and R2 samples had lower nitrogen and protein contents, and higher C/N ratio compared to U for all the 3 species. However, the C/N ratio of CP-R1 was nearly the same as CP-U indicating that for this high protein microalga, the HPAA method resulted in a proportional fractionation of carbohydrates and lipids to proteins. C/N ratio was the highest for all R2 (6.6, 9.2 and 7.2 for CP-R2, TC-R2 and PT-R2 respectively) compared to the other 3 substrates of the respective species. Yet, the ratio for all the substrates was lower than the suggested optimal range of 20-30 for AD (Parkin & Owen, 1986).

Figure 5.1 shows the COD solubility (%) of all the 12 substrates. Among the untreated biomass, CP-U had the lowest COD solubility owing to its recalcitrant cellulosic cell wall that prevents solubilisation of intracellular components. The COD solubility values for CP and TC from this study are comparable to those reported for *Chlorella sp.* and *Tetraselmis sp.* (Bohutskyi et al., 2014). D and R1 substrates of all the 3 species showed a higher COD solubility relative to U substrates as a result of cell disruption. Cell disruption generally improves COD solubility of CP-R1 was lower than CP-D (by 43.5 %), TC-R1 was nearly the same as TC-D, and PT-R1 was higher than PT-D (by 49.6%). R1 fraction is usually enriched in non-protein components. The insoluble cell wall components, mainly remaining in the pellet from the solubilisation step, also end up in this fraction. The lower COD solubility of CP-R1 resulted from the higher content of cell-wall originated insoluble polysaccharides such as cellulose and algaenans (originating from the cell wall) relative to CP-D. The higher COD solubility of PT-R1 relative to PT-D resulted from the higher content of soluble sugars recovered in PT-R1.

TC-R2 and PT-R2 showed lower COD solubility than the respective U and D substrates, and CP-R2 relative to CP-D. This could be attributed to the higher lipid and insoluble polysaccharide contents and possibly the formation of refractory compounds due to Maillard reactions under hydrothermal conditions. Such observations were made previously (Alzate et al., 2012). However, the solubility was 24.2 % higher for CP-R2 relative to CP-U.

#### 5.3.2 Anaerobic digestion

#### 5.3.2.1 Methane yield and methane production efficiency

Experimental methane production curves for all the substrates are shown in Figure 5.2. A majority of total methane was generated during the first 45-50 days of digestion for almost all the substrates except PT-R2. PT-R2 did not produce any methane until day 14 and did not reach the stationary phase at the end of the digestion period unlike other PT substrates. This suggested the presence of refractory compounds formed under hydrothermal conditions as discussed in section 5.3.1. The anaerobic microorganisms needed to acclimatize to this substrate before producing methane.

Figure 5.3 shows the experimental methane yields and methane production efficiency (percentage ratio of experimental to theoretical yield) for all the 12 substrates. The calculated theoretical methane yields are presented in supplementary Table S5.1. For each species, R1 had the least and R2 had the highest theoretical methane yields.

## Experimental methane yield and methane production efficiency for non-fractionated biomass

Among the untreated biomass, experimental methane yield and methane production efficiency were the highest for PT-U ( $224.1 \pm 11.2 \text{ mL/ gVS}$  and 51.4 % respectively). This was unexpected since TC-U had the most fragile cell wall and the highest COD solubility. The

methane yields from this study for the untreated substrates were lower than those reported in literature for Chlorella sp., Tetraselmis sp. and Phaeodactylum tricornutum over a shorter digestion period (Bohutskyi et al., 2014; Ward & Lewis, 2015; Ward, 2015; Zamalloa et al., 2012). The reasons include the lower inoculum to substrate ratio (1:2), nature of the inoculum and the differences in the biochemical composition of the strains used in the current study. Lower methane productivities at lower inoculum to substrate ratios of 1:3 and 1:1 compared to 2:1 were reported previously (Alzate et al., 2012). The methane yields reported for the same species also differed from different studies (Passos et al., 2014). Surprisingly, there was no significant difference in methane yields between TC-U and CP-U, although the COD solubility was higher for the former, and the latter has a more recalcitrant cell wall. A pH drop from 7.0 to 6.4 (which is below the optimal range (Parkin & Owen, 1986)) was observed during the first 3 days of AD of TC-U and reached the optimum range only by day 11. This pH drop during the early phase of AD due to VFA accumulation was responsible for the lower productivity of this substrate. It has been suggested that large inoculum size helps in maintaining pH during batch AD and lower inoculum substrate ratio would result in lower productivity and rate of methane production (Nallathambi Gunaseelan, 1997). Nevertheless, the same I/S ratio used in this study did not result in a drop of pH below the optimum range for any of the other substrates.

For all the 3 species, the disrupted substrates resulted in higher experimental methane yields and better methane production efficiencies than the untreated substrates. These results were in accordance with the fact that cell disruption hydrolyses cell wall polymers and allows access to intracellular components, thereby increasing COD solubility, methane yield and methane production efficiency (González-Fernández et al., 2011). Improvement of biogas

production (by 33 %) and anaerobic degradation rate for high pressure cell disrupted (using French press) *Nannochloropsis salina* was reported previously (Schwede et al., 2011).

#### Experimental methane yield and methane production efficiency for fractionated biomass

R1 substrates from all the 3 species had higher methane yields and methane production efficiency relative to untreated substrates. The easy access to organic compounds by anaerobic bacteria and the slightly higher C/N ratio of R1 substrates were possibly responsible for this. The yield and production efficiency for CP-R1 were higher than CP-U by 57.1% and 59.0%, and by 9.0 % and 24.8% than CP-D. The yield from TC-R1 was higher than that from TC-U by 68.3 % but not significantly different from TC-D. The yield enhancement observed for CP-R1 and TC-R1 in this study was higher than the 37 % reported by Astals et al. (2015) for Scenedesmus sp. residues obtained after protein extraction by free nitrous acid pretreatment method, and the 30.4 % reported for protein extracted residual Spirulina platensis biomass in our previous work (Astals et al., 2015; Parimi et al., 2015a). The methane production efficiency of TC-R1 was much higher relative to both TC-U and TC-D, and reached 81.5%. The yield for PT-R1 was only slightly higher (by 17.4%) than PT-U. However, in terms of methane production efficiency PT-R1 performed extremely well, achieving about 90.2 % of its theoretical yield. This was 75.5 % and 59.1 % higher than the production efficiency of PT-U and PT-D respectively. These results are in accordance with the higher COD solubility observed for PT-R1 relative to PT-U and PT-D.

Among R2 substrates, the yield for CP-R2 was higher than CP-U and CP-D (by 58.5% and 10.3 % respectively). In terms of production efficiency, CP-R2 performed better than CP-U but slightly worse than CP-D. The methane yield for TC-R2 was nearly the same as TC-U, but lower than TC-D (by 31.7 %), and its production efficiency was the least of all TC substrates.

PT-R2, with an extremely low yield and production efficiency, performed the worst among all the substrates in this study. Thus, the LTHT fractionation process benefitted only CP, the species with a highly recalcitrant cell wall. All R2 substrates were enriched in non-protein components, and had the highest C/N ratio and theoretical methane yield among the substrates from the respective species. Yet, they exhibited low production efficiency and COD solubility, possibly due to the higher content of lipids and complex polysaccharides that are not easily hydrolyzed by the anaerobic bacteria, and the presence of refractory compounds formed under hydrothermal conditions as discussed in section 5.3.1. For PT-R2 in particular, the hydrothermal processing conditions were completely detrimental.

# 5.3.2.2 Kinetics of methane production

Table 5.2 shows the kinetic parameters of methane production for all 12 substrates obtained by fitting the experimental data to the modified Gompertz equation. The regression coefficients,  $R^2$  (adjusted) for all the substrates were greater than 0.98 indicating a good agreement of the curve fit with the experimental data. All the predicted parameters were highly significant (p < 0.002). The methane production potentials (P) predicted by the model generally followed the same trend and were not significantly different from the experimental methane yields obtained at the end of the digestion period (reported in section 5.3.2.1). This indicated that the AD process was carried out to near completion for all the substrates.

# Kinetic parameters for non-fractionated biomass

Among the untreated substrates, the predicted methane production potential (P) and the kinetic rate of methane production ( $R_m$ ) were the highest for PT-U. Among CP-U and TC-U, the latter had a lower productivity and rate but also a significantly lower lag phase. This is a clear indication that the AD of TC-U began much earlier than CP-U owing to its relatively less

recalcitrant protein-based cell wall and higher COD solubility. However, the pH drop during the early phase of AD for this substrate inhibited methanogenic microbes, resulting in lower methane productivity and overall rate of digestion. The production potentials and kinetic rates were significantly higher for all D substrates relative to U ones.

#### Kinetic parameters for fractionated biomass

The methane potentials and kinetic rates for all R1 substrates were higher (by 8.6-63.8 % and 59.4 - 169.5 %) than the respective U substrates, with the relative difference following the order TC-R1 > CP-R1 > PT-R1. This is in accordance with previous studies where higher rates were observed for amino acid extracted *Scenedesmus* residues (Ramos-Suárez and Carreras, 2014) and for protein extracted *Spirulina* residues (Parimi et al., 2015a). In this study, a significant drop in lag phase was observed for PT-R1 alone among all R1 (8.4 d from 14.7 d for PT-U and 11.3 d for PT-D), indicating that this substrate was the most readily digested by the anaerobic bacteria.

Among R2 substrates, the predicted methane production potential and rate of methane production for CP-R2 were higher than CP-U (by 38.5 % and 31.3% respectively). The hydrothermal fractionation process that generated CP-R2 resulted in cell wall rupture allowing access of intracellular components by anaerobic bacteria and resulting in higher methane productivity and rate relative to CP-U that had an intact highly recalcitrant cell wall. The production potential of CP-R2 was higher than CP-D (by 19.9 %) due to the higher content of energy rich compounds such as lipids, but rate was lower (by 25.9 %) owing to their slower degradation kinetics (Montingelli et al., 2015). For TC-R2, the production potential and kinetic rate were higher than TC-U (by 21.1 % and 26.9 % respectively) but lower than TC-D (by 22.9 % and 56.6 % respectively), although its COD solubility was lower than both the non-fractionated substrates. This is clearly the result of low production potential and kinetic rate for

TC-U resulting from the VFA accumulation and pH drop as discussed in section 5.3.2.1. The production potential and kinetic rate for PT-R2 were much lower than PT-U (by 71.3 % and 77.0 % respectively) and PT-D (by 73.7 % and 84.9% respectively). PT-R2 showed the longest lag phase (33 days) among all substrates used in this study, which spanned about 45% of the total digestion period. As discussed earlier, the reason for the low production potential and rate, and a very long lag phase of PT-R2 was the possible presence of inhibitory compounds in this substrate.

### 5.3.2.3 Energy output and recovery

Table 5.3 reports the higher heating values (MJ/ kg), the energy output (MJ/ kg) and energy recovery (%) for each of the AD substrates. Among the untreated substrates the energy output and recovery were of the order PT-U > CP-U > TC-U. As is the case with the other AD parameters, the  $E_{output}$  and ER were higher for all D substrates compared to the respective U substrates owing to their higher biomethane yields.

R1 substrates had the least HHV among all other substrates of the respective species, yet their ERs were the highest. The ERs for CP-R1, TC-R1 and PT-R1 were higher than those for CP-U, TC-U and PT-U by 80.7 %, 164.6 % and 93.9 % respectively. This implied that R1 substrates were highly efficient for biomethane production. The E<sub>output</sub> and ER were the highest for PT-R1 among all substrates used in this study.

R2 substrates had the highest HHV among all the other substrates of the respective species owing to their relatively high carbon contents. Yet, TC-R2 and PT-R2 had the least energy recoveries among other substrates of the respective species. On the other hand, CP-R2 had a higher  $E_{output}$  compared to all other CP substrates, and a higher ER (by 30.2 %) relative to CP-U. As expected, PT-R2 had the least ER and hence was the worst substrate for AD.

#### 5.3.3 Protein isolates characterization

Based on the results from section 5.3.2, it is clear that R1 substrates are very efficient for AD. Thus, the protein isolates (designated CP-Prt, TC-Prt and PT-Prt), which are the co-product fractions from the HPAA process that generated R1, were further characterized to prospect potential applications. The protein isolates had high protein content (72.5-75.6 %) and low ash content (4.5-6.9 %) (Data presented in table S5.2 of the supplementary section).

Table 5.4 reports the essential amino acid composition of the untreated biomass (CP-U, TC-U and PT-U) and their protein isolates (CP-Prt, TC-Prt and PT-Prt). The protein isolates had a higher content of most of the essential amino acids compared to untreated biomass of the respective species. In general, the essential amino acid composition of all the protein isolates compared favorably with the conventional protein sources and the contents recommended by World Health Organization/ Food and Agriculture Organization (also reported in Table 5.4 and adopted from (Becker, 2007)). Based on this data, it appears that the isolates could be used as human or animal feed. The use of whole and fractionated microalgal biomass as animal feed supplements is widely reported in literature. For example, algae meal (a combination of oil-extracted microalgae and soybean) were readily consumed by lambs when included upto 60% of the diet dry matter (Stokes et al., 2015). Similar feed trials will validate the applicability of the microalgal protein isolates obtained in this study as animal feed supplements.

## **5.4 Conclusions**

This study evaluated the performance of protein fractionated microalgal biomass obtained by 2 different fractionation methods (HPAA and LTHT) relative to the respective nonfractionated (untreated and HPH cell disrupted) biomass from 3 different species. The results varied significantly across species and treatments. However, for all the species, protein fractionated residues generated by HPAA method (R1) performed the best, in terms of methane yield, production efficiency, predicted production potential, kinetic rate, lag phase and energy recovery. PT-R1 in particular, was the most efficient substrate but the relative difference from its untreated substrate was less pronounced than for the other 2 species. Although the methane yields and rates of methane production for the HPH disrupted substrates (D) were similar to the respective R1 substrates, the latter scored over the former on account of their better methane production efficiency and energy recovery. In addition, the HPAA fractionation process also generated a potentially useful co-product (the protein isolate), that could help in offsetting the costs involved in the fractionation process. Thus, the benefits of the coupled process of protein extraction by the HPAA method and the use of the residual biomass for biomethane production look promising.

On the other hand, CP-R2 was the only one among R2 substrates that performed better than its untreated biomass (CP-U), and slightly better or similar relative to its cell disrupted biomass (CP-D). Thus, AD of the residues from LTHT protein fractionation method seems to be beneficial only for the species with highly recalcitrant cell walls. Yet, the sustainability of the overall process would depend on factors such as input costs, net energy output and the utility of the aqueous co-product fraction generated from this method.

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**Table 5.1** Biochemical composition of all *Chlorella pyrenoidosa* (CP), *Tetraselmis chuii* (TC) and *Phaeodactylum tricornutum* (PT) substrates used in the AD study. U - untreated; D - disrupted; R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method. Values in parentheses represent standard deviations.

Substrate	TS (%)	VS (%)	Ash (%)	N (%)	C (%)	H (%)	S (%)	O (%)	C/N	Protein	tCOD	sCOD
									ratio	(%)	(g/ L)	(g/ L)
CP-U	p.n*	90.4 (0.5)	9.6 (0.8)	9.3 (0.2)	46.8 (1.1)	6.8 (0.9)	1.1 (0.1)	26.4 (1.4)	5.0 (0.0)	58.3 (1.3)	24.4 (0.0)	4.7(0.0)
CP-D	4.2 (0.0)	90.4 (0.5)	9.6 (0.8)	9.3 (0.2)	46.8 (1.1)	6.8 (0.9)	1.1 (0.1)	26.4 (1.4)	5.0 (0.0)	58.3 (1.3)	24.4 (0.0)	8.8 (0.0)
CP-R1	3.2 (0.0)	82.2 (0.6)	17.8 (0.6)	8.0 (0.3)	41.5 (2.1)	6.0 (0.4)	1.0 (0.0)	25.7 (2.8)	5.2 (0.4)	50.0 (1.4)	18.5 (1.0)	3.8 (0.1)
CP-R2	5.7 (0.3)	92.9 (3.6)	7.1 (2.0)	8.5 (0.2)	55.9 (0.3)	7.0 (0.0)	0.8 (0.0)	20.7 (0.1)	6.6 (0.5)	53.1 (1.3)	32.0 (0.1)	7.7 (0.0)
TC-U	p.n*	84.1 (0.8)	15.9 (0.8)	7.4 (0.1)	42.7 (0.4)	6.2 (0.1)	1.2 (0.1)	27.4 (0.5)	6.5 (0.0)	46.3 (0.6)	19.4 (0.0)	7.4 (0.0)
TC-D	4.0 (0.0)	84.1 (0.8)	15.9 (0.8)	7.4 (0.1)	42.7 (0.4)	6.2 (0.1)	1.2 (0.1)	27.4 (0.5)	6.5 (0.0)	46.3 (0.6)	19.4 (0.0)	8.5 (0.0)
TC-R1	3.1 (0.0)	74.8 (0.6)	25.2 (0.6)	3.7 (0.0)	31.5 (0.5)	5.0 (0.1)	0.9 (0.0)	33.7 (0.6)	8.4 (0.1)	23.4 (0.1)	14.1 (0.2)	6.2 (0.1)
TC-R2	5.3 (0.2)	87.9 (2.0)	12.1 (3.6)	5.8 (0.2)	52.9 (2.0)	6.6 (0.1)	0.9 (0.3)	21.7 (1.5)	9.2 (1.2)	36.6 (1.5)	21.0 (0.4)	3.9 (0.0)
PT-U	p.n*	84.7 (1.1)	15.3 (1.1)	8.7 (0.3)	44.7 (0.4)	6.5 (0.3)	1.3 (0.1)	23.4 (0.4)	5.2 (0.2)	54.4 (1.9)	25.3 (0.0)	8.1 (0.1)
PT-D	4.1 (0.1)	84.7 (1.1)	15.3 (1.1)	8.7 (0.3)	44.7 (0.4)	6.5 (0.3)	1.3 (0.1)	23.4 (0.4)	5.2 (0.2)	54.4 (1.9)	25.3 (0.0)	10.4 (0.9)
PT-R1	2.7 (0.0)	66.9 (0.5)	33.0 (0.5)	4.6 (0.3)	29.5 (1.7)	5.1 (0.3)	1.0 (0.0)	26.8 (2.2)	6.4 (0.0)	28.8 (1.9)	18.6 (0.1)	11.5 (0.8)
PT-R2	6.6 (1.8)	86.7 (1.87)	13.3 (1.87)	7.7 (0.5)	55.5 (1.7)	6.7 (0.3)	0.9 (0.1)	15.8 (2.5)	7.2 (0.3)	48.1 (3.1)	37.9 (1.1)	12.0 (0.0)

\*p.n. - prepared as needed

**Table 5.2** Methane production potential (P), kinetic rate of methane production ( $R_m$ ) and lag phase ( $\lambda$ ) for methane production from all substrates. U - untreated; D - disrupted; R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method. Values in parentheses represent standard errors.

Substrate	P (mL /gVS)	$R_m (mL/gVS/d)$	λ (d)	$\mathbf{R}^2$ (adj)
CP-U	171.8 (6.4)	4.8 (0.5)	17.5 (1.6)	0.9891
CP-D	238.0 (7.6)	8.5 (1.0)	16.9 (1.6)	0.9857
CP-R1	252.3 (5.8)	9.7 (1.0)	15.8 (1.2)	0.9913
CP-R2	285.5 (17.6)	6.3 (0.6)	17.6 (1.8)	0.9891
TC-U	152.3 (10.1)	2.6 (0.2)	8.0 (1.8)	0.9855
TC-D	239.2 (8.6)	7.6 (0.8)	17.0 (1.8)	0.9854
TC-R1	249.4 (10.1)	7.0 (0.7)	11.0 (1.9)	0.9800
TC-R2	184.5 (15.9)	3.3 (0.3)	13.1 (2.2)	0.9810
PT-U	242.1 (6.9)	6.1 (0.5)	14.7 (1.1)	0.9953
PT-D	265.1 (2.0)	9.3 (0.5)	11.3 (0.4)	0.9991
PT-R1	263.0 (1.8)	9.7 (0.5)	8.4 (0.4)	0.9991
PT-R2	69.6 (12.0)	1.4 (0.2)	33.3 (1.8)	0.9840

Substrate	HHV (MJ/ kg)	E <sub>output</sub> (MJ/ kg)	ER (%)
CP-U	20.7	5.5	26.8
CP-D	20.7	8.0	38.5
CP-R1	18	8.7	48.4
CP-R2	25.2	8.8	34.9
TC-U	18.4	4.9	26.4
TC-D	18.4	7.9	42.9
TC-R1	11.7	8.2	69.9
TC-R2	23.5	5.4	22.9
PT-U	20.3	7.7	38.0
PT-D	20.3	9.1	44.6
PT-R1	12.4	9.1	73.7
PT-R2	25.6	1.9	7.5

**Table 5.3** Higher heating value (MJ/ kg), energy output (MJ/ kg) and energy recovery (%) for allsubstrates. U - untreated; D - disrupted; R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method.

Amino acid	CP-U	CP-Prt	TC-U	TC-Prt	PT-U	PT-Prt	WHO /FAO*	Egg*	Soybean*
Thr	5.4	5.5	5.4	5.5	5.2	5.4	n.r	5.0	4.0
Val	6.1	6.2	5.6	5.9	5.5	5.8	5.0	7.2	5.3
Ile	4.2	4.4	4.2	4.6	4.6	5.2	4.0	6.6	5.3
Leu	9.3	9.6	8.8	9.7	7.8	9.1	7.0	8.8	7.7
Phe	5.9	6.1	5.9	6.6	5.3	6.0	6.0	5.8	5.0
His	2.0	1.9	1.9	2.1	2.0	2.1	n.r	2.4	2.6
Lys	6.4	5.8	6.2	5.5	5.9	6.2	5.5	5.3	6.4
Met	2.7	2.6	3.0	2.9	2.9	3.0	3.5	3.2	1.3
Trp	2.6	2.5	2.3	2.8	2.1	2.5	1.0	1.7	1.4

**Table 5.4** Comparison of essential amino acid composition of the protein isolates (Prt) obtained from high pressure alkali-acid process, untreated biomass (U), conventional protein sources and World Health Organization/ Food and Agriculture Organization (WHO/ FAO) recommendation.

n.r - not reported

\* indicates data adopted from Becker, 2007



**Figure 5.1.** COD solubility ([sCOD/tCOD]\*100) (%) for the non-fractionated and proteinfractionated residue substrates. U - untreated; D - disrupted; R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method. Error bars represent standard deviations.







**Figure 5.2.** Methane production curves for non-fractionated (U - untreated and D - disrupted) and protein-fractionated residue (R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method) substrates: (a) *Chlorella pyrenoidosa*, (b) *Tetraselmis chuii* and (c) *Phaeodactylum tricornutum*. Error bars represent standard deviations.



**Figure 5.3.** (a) Experimental methane yield (mL/ gVS) and (b) methane production efficiency (%) of non-fractionated (U - untreated and D - disrupted) and protein fractionated residue (R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extracted residue from low temperature hydrothermal method) substrates. Error bars represent standard deviations.

## CHAPTER 6

### CONCLUSIONS AND RECOMMENDATIONS

#### **6.1 Conclusions**

This dissertation was aimed at contributing to the technical know-how on microalgae/ cyanobacteria as a feedstock for the simultaneous generation of biofuels and co-products in a combined biorefinery concept. More specifically, an integrated process involving microalgal protein fractionation to generate protein isolates/extracts (co-products), and biofuel production (via anaerobic digestion (AD) and hydrothermal liquefaction (HTL)) from the residual biomass was investigated. The limitation to microalgal AD and HTL arising from its high protein content was overcome by protein fractionation/ isolation. The results from the investigations were reported as three different studies. The first study involved the development and optimization of a process for microalgal/ cyanobacterial protein extraction. The optimized process was able to generate a high quality protein isolate at a reasonably high yield and a residual biomass that had a more favorable biochemical composition for biofuel feedstock applications than the original (untreated) biomass. The second study investigated the performance of the residual biomass obtained after protein fractionation using the method developed in part 1 as a feedstock for biomethane production via AD and biocrude oil production via HTL. The results indicated that the use of the residual biomass benefitted both AD and HTL processes. The third part studied the methane production from microalgal residues obtained after protein fractionation from three different microalgae using two different methods (the method similar to the one described in the first study of this dissertation and a low temperature hydrothermal fractionation method). The residues from the former method resulted in higher methane yields and methane production efficiencies relative to the non-fractionated biomass as well as the residues obtained from the latter method. The research work presented in this dissertation is very useful for scientists and engineers who are interested in the development of integrated microalgal biorefineries capable of processing wet microalgae biomass to biofuels and obtaining protein extracts for nutritional supplementation.

The specific conclusions from each of the studies are presented in the following sections.

6.1.1 Optimization of protein extraction from Spirulina platensis to generate a potential coproduct and a biofuel feedstock with reduced nitrogen content

This study developed a process for extraction of microalgal/cyanobacterial proteins at a high yield and generation of a residual biomass fraction with a biochemical composition better suited for biofuel applications in comparison to original untreated biomass. The process comprised of high pressure homogenization based cell disruption of *Spirulina platensis* cells, followed by solubilisation under alkaline pH conditions and a subsequent precipitation under acidic pH conditions. The following were the conclusions drawn from this study:

- 1. The most significant parameters that influenced protein extractability were cell disruption efficiency, biomass concentration, pH and precipitation time.
- 2. The optimum process conditions for protein extraction from S. platensis were :
  - a. Cell disruption by high pressure homogenization: 2 passes at 103.4 MPa
  - b. Solubilisation step: pH: 11.36; solubilisation time: 35 min; and biomass concentration:
    3.6 % (w/w) solids.
  - c. Precipitation step: pH: 4.01; precipitation time: 60 min
- 3. At the optimized conditions the protein isolate was obtained at a high yield (60.7%).

- 4. The protein isolate had a better nutritional composition than original *S. platensis*. It had a higher protein content (80.6 %), was enriched in essential amino acids and  $\gamma$ -linolenic acid, and had lower ash and mineral contents.
- 5. The residual biomass with lower nitrogen and higher non-protein composition was more promising as a biofuel feedstock than original (untreated) *S. platensis* biomass.

6.1.2 Biomethane and biocrude oil production from protein extracted residual Spirulina platensis

This study investigated the performance of residual biomass after protein fractionation of *Spirulina platensis* using the method optimized in the previous study (6.1.1), as feedstock for AD and HTL. The specific conclusions from this study are listed below:

- 1. The protein extracted residual biomass (PERB) had a higher methane yield than the untreated biomass (by 30.4 %), and a higher kinetic rate of methane production relative to both the original and cell disrupted *S. platensis* biomass substrates (by 161 % and 38.9 % respectively).
- 2. The biocrude oil generated from HTL of PERB was better in quality than that generated from originial biomass owing to the slightly reduced (by 11.4 %) nitrogen content, and the presence of a higher number of long chain hydrocarbons and fatty acids and a lower number of nitrogenous compounds relative to the latter. However the yield of the former was lower than the latter.
- 3. PERB as a biofuel feedstock performed better in AD process than HTL owing to the higher biofuel yield, energy output and energy recovery.

6.1.3 Evaluation of microalgal biomass residues from protein fractionation processes as substrates for anaerobic digestion

This study evaluated protein extracted residues from three different micoalgal species – a freshwater green microalga (*Chlorella pyrenoidosa*), a marine green microalga (*Tetraselmis chuii*) and a marine diatom (*Phaeodactylum tricornutum*), obtained by two different protein fractionation processes as feedstocks for AD. The experimental methane yield, production efficiency, production potential, kinetic rate of methane production, lag phase time and energy recovery were determined. The specific conclusions from this study were:

- The experimental methane yields (253.3-265.4 mL/ gVS), methane production efficiencies (60.8-90.2 %) and energy recoveries (48.4-73.7 %) for the protein fractionated/extracted residues generated by the high pressure alkali-acid (HPAA) method were higher than the non-fractionated biomass.
- 2. The protein fractionated/extracted residue generated from low temperature hydrothermal (LTHT) method for *Chlorella pyrenoidosa* (CP) alone resulted in a higher methane yield and production potential relative to both its non-fractionated biomass substrates.
- 3. The methane yield, production efficiency, rate and energy recovery enhancement by protein extracted residues relative to the untreated biomass was species specific.
- 4. The protein isolates obtained from HPAA method were of high quality in terms of protein and essential amino acid contents, and hence look promising for human food or animal feed applications.

#### **6.2 Recommendations**

This dissertation advances the knowledge on microalgal biomass fractionation to extract protein co-products and biofuel/ biofuel intermediate production via wet processing technologies. The protein co-products could be used as nutritional supplements for human beings or as feed for animals, and the residual biomass could be used as feedstock to generate biofuels/ biofuel intermediates that could be used (directly or after upgrading) for electricity generation, transportation and other domestic and industry applications. Thus, this work would be of great value to scientists, engineers and biofuel industry professionals who are working towards solving the global problems of food insecurity and energy crisis. However, further studies in this direction are necessary to ensure the scalability, technical feasibility and economic sustainability of the proposed integrated processes. Specific recommendations for future studies include:

- 1. Implementation of protein extraction processes in continuous mode on a larger scale to acquire data on the technical feasibility of the process and help identify any bottlenecks that can be resolved.
- 2. Evaluation of the performance of protein fractionated residues as feedstock for AD and HTL in continuous processes as opposed to batch processes used in the current study, in order to obtain data for feasibility analysis.
- 3. A detailed nutritional quality analysis and feed trials for the protein isolate to validate its proposed application as a food or feed grade nutritional supplement.
- 4. Investigation of applications for the aqueous, gas and solid fractions from HTL process and the digestate from AD process to improve process sustainability.
- 5. Techno-economic and life cycle analysis of the integrated process of protein extraction and biofuel production.

# APPENDIX A

# SUPPLEMENTARY DATA FOR CHAPTER 3

**Table S3.1** Box-Behnken design of protein solubilisation experiments and the experimental

 response (protein recovery in alkali supernatant) at each set of the process conditions.

Variable		Coded variable	C	oded variable	levels	
			-1	0	1	
pН		$\mathbf{X}_1$	10.5	11.25	12	
Solubilisa	tion time (min)	$X_2$	10	35	60	
Biomass c	concentration(% w/w)	$X_3$	2	6	10	
Run		Coded variables		Pr re	Protein recovery (%)	
	$\mathbf{X}_{1}$	$\mathbf{X}_{2}$	<b>X</b> <sub>3</sub>			
1	-1	0	-1	92	.30	
2	0	1	-1	91	.10	
3	0	-1	-1	90	.12	
4	1	0	-1	95	.30	
5	1	1	0	87	.63	
6	1	-1	0	88	.33	
7	0	0	0	93	.20	
8	0	0	0	95	.60	
9	-1	1	0	90	.73	
10	0	0	0	90	.30	
11	-1	-1	0	89	.24	
12	0	1	1	76	.85	
13	1	0	1	64	.87	
14	-1	0	1	76	.15	
15	0	-1	1	68	.09	

Variable		Coded variable		Coded variable levels		
			-1	0	1	
pН		$\mathbf{X}_1$	3	4	5	
Precipitation	time (min)	$X_2$	10	35	60	
_				Pro	tein recovery	
Run		Coded va	<b>Coded variables</b>		(%)	
	$\mathbf{X}_{1}$		$\mathbf{X}_2$			
1	-1		-1	67.	0	
2	-1		0	68.3		
3	-1		1	70.:	5	
4	0		-1	73.:	5	
5	0		0	74.:	5	
6	0		0	72.	6	
7	0		1	76.	1	
8	1		-1	70.2	2	
9	1		0	68.	1	
10	1		1	71.	8	

Table S3.2 Box-Behnken design of protein precipitation experiments and the experimental response (protein recovery in acid pellet) at each set of the process conditions

Sample	Total Amino sugars (%)	NAMA (%)	GlcNAc (%)	<b>DAP</b> (%)
Protein isolate	0.35	0.08	0.27	0.74
Residual biomass	1.69	0.59	1.10	0.98

**Table S3.3** Contents (% w/w) of the PG amino sugars and DAP in the protein isolate and the residual biomass fractions

		Original
Elements	Protein isolate	S. platensis <sup>a</sup>
Al	13.2	83.2
В	< 0.50	n.a.
Ca	<2.50	1165.4
Cd	< 0.25	0.1
Cr	< 0.25	3.3
Cu	< 0.25	4.7
Fe	20.1	568.8
Κ	2270.5	19821
Mg	< 0.75	4247.8
Mn	< 0.25	36.6
Мо	< 0.25	n.a.
Na	735.5	730.7
Ni	< 0.50	0.4
Р	168.4	11823.3
Pb	<1.25	0.1
S	318.4	n.a.
Si	24.7	206
Zn	< 0.25	12.3

Table S3.4 Mineral composition of the protein isolate and the original S. platensis biomass

<sup>a</sup> Data obtained from Jena et.al., 2011



Figure S3.1. Schematic of the protein isolation process



**Figure S3.2.** Comparison of different pretreatments for protein solubilisation based on protein recovery (%) in the alkali supernatant



**(C)** 



**Figure S3.3.** Microscope images of freeze dried *S. platensis* cells resuspended in DI water (at 400 times magnification) (A) before; and after cell disruption using (B) Ultrasonication (20 % maximum power, 60 min) (C) High pressure homogenization (2 passes, 103.4 MPa).



1 – Ladder; 2 – Freeze dried *S.plantensis* resuspended in DI water; 3- *S. platensis* biomass subjected to high pressure homogenization; 4- Alkali Pellet; 5- Acid pellet/ protein isolate; 6- Acid Supernatant.; 7- Residual biomass

**Figure S3.4.** SDS-PAGE analysis of the feed and product fractions of the *S. platensis* protein isolation process carried out at the RSM optimized conditions (Alkali step: (pH: 11.38, solubilisation time: 35.3 min, biomass concentration: 3.61 %); Acid step: (pH: 4.01, precipitation time: 60 min)).

# APPENDIX B

# SUPPLEMENTARY DATA FOR CHAPTER 4

**Table S4.1** Examples of compounds present in the biocrude oil from the original biomass (ORIB biocrude) and that from the protein extracted residual biomass (PERB biocrude) which may be classified into each of the listed compound classes.

Compound class	Abbreviation	ORIB biocrude	PERB biocrude
Long chain hydrocarbons (C10-C20)	LHC	Heptadecane	Pentadecane; hexadecane; heptadecane
Aromatic hydrocarbons	AHC	Styrene; Toluene	Styrene; Toluene
Nitrogenated heterocyclic compounds	NHCC	Octanoic acid, morpholide; 2-Methyl-1- ethylpyrrolidine	Pyrrolidine, 1-methyl-; Piperidine; 1- ethyl-; pyrazine, methyl-
Long chain aliphatic amides	LAA	Hexadecanamide; N- Methyldodecanamide; N,N-Dimethyldecanamide	none
Other aliphatic nitrogenous compounds	ANC	3-Cyano-2- methoxypropionic acid, methyl ester	3-Cyano-2- methoxypropionic acid, methyl ester
Ketones	Ket	none	2-Cyclopenten-1-one; 2,3,4-trimethyl-
Phenolics	Phe	none	Phenol
Fatty acids	FA	none	n-Hexadecanoic acid; 6- Octadecenoic acid
Oxygenates	Оху	2-Bornanol, 2-methyl-; Pentanedioic acid, 3-oxo-, dimethyl ester	1-Butanol, 2-methyl-

**Table S4.2** Major compounds (representing GC-MS chromatogram peaks with areas > 3%) in HTL biocrude oil generated from original biomass (ORIB biocrude) and that from protein extracted residual biomass (PERB biocrude).

		<b>ORIB</b> biocrude		PERB biocrude			
	RT		Area	RT		Area	
S.No	(min)	Compound	(%)	(min)	Compound	(%)	
1	45.2	Octanoic acid, morpholide	14.4	30.5	Heptadecane	8.5	
2	40.1	Hexadecanamide	12.4	25.9	Pentadecane	8.2	
3	40.6	N- Methyldodecanamide	11.2	4.0	Acetamide, N-(3,5- dichlorophenyl)-2-(1- pyrrolidinyl)-	8.0	
4	41.3	N,N- Dimethyldecanamide	8.3	11.9	Conhydrin	7.5	
5	43.5	3H-1,2,4-Triazole-3- thione, 2,4-dihydro-4- methyl-	7.4	36.2	n-Hexadecanoic acid	5.3	
6	36.1	l-Proline, N- allyloxycarbonyl-, hexyl ester	4.9	6.4	Piperidine, 1-ethyl-	4.4	
7	43.2	cis-11-Eicosenamide	4.7	8.0	Styrene	4.3	
8	46.3	Hexadecanoic acid, pyrrolidide	4.3	12.6	Phenol	3.6	
9	22.2	2-Pyridineacetic acid, hexahydro-1-methyl-	3.8	39.4	6-Octadecenoic acid	3.5	
10	30.4	Heptadecane	3.5	11.5	Pyrrolidine, 1-[2-(4- bromophenoxy)ethyl]-	3.1	
11	15.4	2-Methyl-1- ethylpyrrolidine	3.1	6.5	3-Cyano-2- methoxypropionic acid, methyl ester	3.0	

Elements	ORIB ACP (mg L <sup>-1</sup> )	PERB ACP (mg L <sup>-1</sup> )	S. platensis <sup>a</sup> biomass
			$(mg L^{-1})$
Al	13.2	<1.25	83.2
В	<0.50	<0.50	n.d.
Ca	<2.50	24.8	1165.4
Cd	<0.25	<0.25	0.1
Cr	<0.25	<0.25	3.3
Cu	<0.25	<0.25	4.7
Fe	20.1	26.5	568.8
К	2270.5	4430.5	19821
Mg	<0.75	<0.75	4247.8
Mn	<0.25	<0.25	36.6
Мо	<0.25	<0.25	n.d.
Na	735.5	12357.5	730.7
Ni	<0.50	<0.50	0.4
Р	168.4	377.5	11823.3
Pb	<1.25	<1.25	0.1
S	318.4	323.4	n.d.
Si	24.7	34.9	206
Zn	<0.25	<0.25	12.3

**Table S4.3** Mineral composition of ACPs from original biomass (ORIB ACP) and that from protein extracted residual biomass (PERB ACP) compared to *S. platensis* biomass

<sup>a</sup> Data obtained from Jena et al. (2011). n.d. : not determined

# APPENDIX C

## SUPPLEMENTARY DATA FOR CHAPTER 5

**Table S5.1** Theoretical methane yields (mL/ gVS) of all substrates. U - untreated; D - disrupted;R1 - protein extracted residue from high pressure alkali-acid method and R2 - protein extractedresidue from low temperature hydrothermal method.

Substrate	Theoretical methane (mL/ gVS)
CP-U	475.6
CP-D	475.6
CP-R1	416.4
CP-R2	593.4
TC-U	430.8
TC-D	430.8
TC-R1	291.5
TC-R2	567.0
PT-U	436.1
PT-D	464.1
PT-R1	294.4
PT-R2	603.8

Biomass	N (%)	Protein (%)	Ash (%)
CP-Prt	12.1 (0.0)	75.6 (0.2)	6.5 (2.4)
TC-Prt	11.9 (0.1)	74.4 (0.5)	6.9 (1.3)
PT-Prt	11.6 (0.3)	72.5 (2.0)	4.5 (0.7)
TC-Prt PT-Prt	11.9 (0.1) 11.6 (0.3)	74.4 (0.5) 72.5 (2.0)	6.9 (1.3) 4.5 (0.7)

**Table S5.2** Nitrogen, protein and ash contents of the protein isolates (Prt).



**Figure S5.1.** Microscope images (400x) for undisrupted and high pressure homogenizer disrupted *Chlorella pyrenoidosa*, *Tetraselmis chuii* and *Phaeodactylum tricornutum*